

**EVALUACIÓN DE LA CONTAMINACIÓN DE
SUELOS Y AGUAS POR PESTICIDAS EN ZONAS DE
VIÑEDO: DIAGNÓSTICO Y DESARROLLO DE
ESTRATEGIAS FISICOQUÍMICAS DE
PREVENCIÓN Y/O CONTROL**



ALBA ÁLVAREZ MARTÍN

TESIS DOCTORAL 2016



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DEPARTAMENTO DE
QUÍMICA INORGÁNICA

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Trabajo presentado por la Licenciada Alba Álvarez Martín para optar al grado de
Doctora en Química, dentro del programa de Doctorado en Química de la
Universidad de Salamanca.

Salamanca, 12 de Febrero de 2016

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CERTIFICAN:

Que la Licenciada en Química Alba Álvarez Martín ha realizado en los laboratorios del Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), bajo su dirección, el trabajo para optar al grado de Doctor en Química que presenta con el título:

“Evaluación de la contaminación de suelos y aguas por pesticidas en zonas de viñedo: diagnóstico y desarrollo de estrategias fisicoquímicas de prevención y/o control”

Autorizando a la presentación de dicho trabajo en la modalidad de compendio de artículos científicos.

Y para que así conste, firman el presente certificado en Salamanca, a 12 de Febrero de dos mil dieciséis.

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El presente trabajo **“Evaluación de la contaminación de suelos y aguas por pesticidas en zonas de viñedo: diagnóstico y desarrollo de estrategias fisicoquímicas de prevención y/o control”**, ha sido realizado en el Dpto. de Procesos de Degradación del Medio Ambiente y su Recuperación del Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), bajo la dirección de la Prof. Dra. M^a Jesús Sánchez Martín y la Dra. M^a Sonia Rodríguez Cruz.

La realización del mismo ha sido posible gracias a una beca FPI (BES-2011-047811) del Ministerio de Economía y Competitividad (MINECO) y la investigación ha estado asociada al proyecto: **“Evaluación de la contaminación de suelos y aguas por pesticidas en zonas de viñedo de la Rioja: diagnóstico y desarrollo de estrategias fisicoquímicas de prevención y/o control” (AGL2010-15976/AGR)** subvencionado por la Dirección General de Investigación del Ministerio de Ciencia e Innovación.



La presente memoria titulada **“Evaluación de la contaminación de suelos y aguas por pesticidas en zonas de viñedo: diagnóstico y desarrollo de estrategias fisicoquímicas de prevención y/o control”**, elaborada por la licenciada Alba Álvarez Martín y que constituye su Tesis Doctoral para optar al grado de Doctor en Química, ha sido redactada en el formato de compendio de artículos originales de investigación publicados en revistas científicas de prestigio internacional e indexadas en la edición científica del *Journal Citation Reports*.

Y para que así conste se recogen a continuación los tres artículos originales de investigación requeridos, su título, autores y afiliación de los mismos, junto con la referencia completa de la revista científica donde fueron publicados:

Effect of different rates of spent mushroom substrate on the dissipation and bioavailability of cymoxanil and tebuconazole in an agricultural soil

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Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC). Cordel de Merinas 40-52, 37008 Salamanca, Spain

Application of a biosorbent to soil: a potential method for controlling water pollution by pesticides

Environment Science and Pollution Research. 2016. (Publicado on line 1 Febrero 2016) DOI 10.1007/s11356-016-6132-4

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Pesticides and degradation products in groundwaters from a vineyard region: optimization of a multi-residue method based on SPE and GC-MS.

Journal Separation Science. 2012. 35, 3492-3500

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Otros artículos y manuscritos adicionales incluidos en esta tesis son:

Occurrence of pesticides and some of their degradation products in waters in a Spanish wine region

Journal of Hydrology. 2013. 486, 234-245

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Development of a procedure for multiresidue analysis of pesticides in vineyard soils and its application to real samples

Journal of Separation Science. 2014. 37, 2215-1124

Eva Pose-Juan, Eliseo Herrero-Hernández, **Alba Álvarez-Martín**, María J. Sánchez-Martín, M. Sonia Rodríguez-Cruz

Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC). Cordel de Merinas 40-52, 37008 Salamanca, Spain

Changes in activity and structure of the soil microbial community after application of azoxystrobin or pirimicarb and an organic amendment to an agricultural soil

Applied Soil Ecology. 2015. Enviado. Ref. No.: APSOIL-D-15-00836

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Leaching of two fungicides in spent mushroom substrate amended soil: Evaluation of different factors influencing their mobility

Artículo en preparación

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La Comisión Académica del Doctorado en Química, reunida en sesión ordinaria el día 11 de febrero de 2016, acordó autorizar la presentación de la memoria de Tesis Doctoral titulada **“Evaluación de la contaminación de suelos y aguas por pesticidas en zonas de viñedo: diagnóstico y desarrollo de estrategias fisicoquímicas de prevención y/o control”**, presentada por la licenciada Alba Álvarez Martín, realizada bajo la dirección de la Dra. M^a Jesús Sánchez Martín y la Dra. M^a Sonia Rodríguez Cruz, en el Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), **en formato de compendio de artículos /publicaciones**

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Jose Luis Usero García

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1. INTRODUCCIÓN

1. INTRODUCCIÓN GENERAL

1.1. PESTICIDAS: TIPOS, CONSUMO, USOS EN AGRICULTURA

Se define **pesticida** como aquella sustancia que se destina a combatir plagas o a controlarlas. Su origen puede ser natural o sintético y su naturaleza orgánica o inorgánica.

La **historia del desarrollo y utilización** de los pesticidas es fundamental para entender cómo y por qué pueden representar una amenaza para el medio ambiente en los sistemas acuáticos y terrestres, y por qué esta amenaza está disminuyendo en los países desarrollados, mientras que continúa siendo un problema en muchos países en desarrollo (Tabla 1).

Tabla 1. Cronología del desarrollo de los pesticidas (FAO, 2015)

| Período | Ejemplo | Fuente | Características |
|------------------|---|--|---|
| 1800-1945 | Primeros pesticidas orgánicos, nitrofenoles, clorofenoles, creosota, naftaleno, aceites de petróleo | Química orgánica, productos derivados de la elaboración de gas de carbón, etc. | Frecuentemente carecían de especificidad y eran tóxicos para el usuario o para organismos que no eran los destinatarios |
| 1945-1955 | Productos orgánicos clorados, DDT, HCCH, ciclodieno, clorados | Síntesis orgánica | Persistentes, buena selectividad, buenas propiedades agrícolas |
| 1955-1970 | Inhibidores de la colinesterasa, organofosforados, carbamatos | Síntesis orgánica, buena utilización de las relaciones estructura-actividad | Menor persistencia, cierta toxicidad para el usuario, algunos problemas ambientales |
| 1970-1985 | Piretroides sintéticos, pesticidas biológicos | Perfeccionamiento de las relaciones estructura-actividad, nuevos sistemas de selección de objetivos | Cierta falta de selectividad, resistencia, costes y persistencia variable |
| 1985-2015 | Organismos obtenidos por la ingeniería genética | Transferencia de genes para pesticidas biológicos a otros organismos. Alteración genética de las plantas | Posibles problemas con mutaciones y fugas, perturbación de la ecología microbiológica, |

Un factor decisivo de la Revolución Verde ha sido el desarrollo y aplicación de pesticidas para combatir gran variedad de plagas de insectos, hongos y malas hierbas, que disminuirían el volumen y calidad de la producción alimentaria. El uso de pesticidas coincide con la "era química", que ha transformado la sociedad desde el década de 1950. En lugares donde se practica el monocultivo intensivo, los pesticidas constituyen el método habitual de lucha contra las plagas. Según el Convenio de Estocolmo sobre Contaminantes Orgánicos Persistentes (Decisión 2006/507/CE) 9 de los 12 compuestos clasificados como más peligrosos y persistentes son pesticidas.

Atendiendo a su **naturaleza química** los pesticidas pueden clasificarse en inorgánicos y orgánicos, lo cual aporta información sobre su posible toxicidad. Los primeros no plantean, en general, una problemática importante desde el punto de vista de su toxicidad y evolución en el suelo.

De acuerdo con su **acción biológica específica** los pesticidas pueden clasificarse en: insecticidas (control de plagas de insectos), herbicidas (elimina malas hierbas), fungicidas (impide el crecimiento y elimina hongos), rodenticidas (elimina o repele roedores), acaricidas (elimina y previene la presencia o acción de los ácaros), bactericidas (elimina bacterias), etc.

Dada la gran cantidad de familias químicas implicadas, la clasificación de los pesticidas resulta difícil. Un recurso útil es hacer una clasificación mixta por ambos criterios, naturaleza química y acción biológica (Tabla 2).

Tabla 2. Clasificación de los pesticidas en función de su naturaleza química y organismo que controlan

| Herbicidas | Insecticidas | Fungicidas |
|-----------------------------|---------------------|--------------------|
| Carbamatos / Tiocarbamatos | | |
| Dinitroanilinas | Carbamatos | Anilinopiridinas |
| Fenoxiácidos | Organoclorados | Azoles / Triazoles |
| Piridínicos / Bipiridínicos | Organofosforados | Benzimidazoles |
| Organoclorados | Piretroides | Carboxiamidas |
| Triazinas | | Fenilamidas |
| Ureas sustituidas | | |

La gran mayoría de los pesticidas aplicados en la **agricultura** son compuestos sintéticos que actúan sobre procesos metabólicos concretos. Los fungicidas actúan en procesos biosintéticos, los insecticidas interfieren en los impulsos nerviosos y los herbicidas actúan sobre la fotosíntesis (Muñoz Leoz, 2012).

Los **fungicidas** son sustancias químicas de origen natural o de síntesis para el tratamiento de las enfermedades producidas por hongos. Los hay que destruyen el micelio y las esporas del hongo, que detienen su desarrollo e impiden la geminación de las esporas, o que impiden la reproducción del hongo. Por su modo de acción se clasifican en: preventivos (impiden la germinación de esporas) y curativos (penetran en los tejidos vegetales y detienen el crecimiento del micelio del hongo).

La mayoría de los **insecticidas** actúan dañando el sistema nervioso de los insectos, afectando directamente a las neuronas, o bien a la transmisión de impulsos nerviosos entre las neuronas. Dentro de la gran variedad de compuestos con acción insecticida, se pueden destacar los organofosforados, carbamatos, piretroides, insecticidas microbiológicos y por último los insecticidas biotecnológicos, denominados de 3ª generación.

Los **herbicidas** son productos químicos que controlan las malas hierbas. El problema de muchos herbicidas es que, al igual que eliminan la planta no deseada, pueden hacerlo con la cultivada o provocarle daños. Por ello requieren ser aplicados correctamente y en los momentos adecuados, teniendo en cuenta el estado del cultivo. Se pueden clasificar según la vegetación afectada (total o selectiva), según el momento de aplicación (presembrado, preemergencia y postsembrado), según el modo de acción (persistentes o residuales, de contacto, hormonales y sistémicos o de translocación interna).

Para que un pesticida alcance un amplio uso en la práctica agrícola, debe reunir determinadas condiciones básicas (Sánchez-Martín y Sánchez-Camazano, 1984) como:

- ✓ Efectividad: debe ser efectivo en la destrucción de la plaga contra la que actúa.
- ✓ Selectividad: debe combatir únicamente los organismos dañinos sin perjudicar a la flora o a la fauna.
- ✓ Economía: la utilización de un pesticida debe producir unos beneficios que

superen el gasto que supone su utilización.

- ✓ Seguridad: no debe ser tóxico para las plantas útiles al hombre ni constituirse en un peligro para la salud del hombre ni de los animales domésticos.
- ✓ Estabilidad: debe conservar su capacidad de acción durante un tiempo suficiente.

España ha sido tradicionalmente un país con un alto consumo de pesticidas y con una tendencia al alza en ese consumo. Así por ejemplo, las ventas y el consumo de pesticidas crecieron en más de 400 millones de euros entre 1993 y 2003. Según datos de las propias industrias que comercializan pesticidas en España, agrupadas en la Asociación Empresarial para la Protección de las Plantas (AEPLA, 2013) las ventas de estos productos en los últimos años han superado los 600 millones de euros anuales. Los datos de la European Crop Protection Agency (ECPA, 2014) muestran a España como uno de los mayores consumidores europeos de pesticidas, llegando a consumir unas 40000 toneladas de pesticidas al año en la agricultura y horticultura, solo contando el principio activo.

En 2013 los fungicidas y bactericidas fueron las sustancias activas más utilizadas representando un 45.3 % del total de sustancias comercializadas (32400 toneladas) (MAGRAMA, 2013). En el otro extremo se encuentran los insecticidas y acaricidas con 9.7 % (6909 toneladas) (Figura 1).

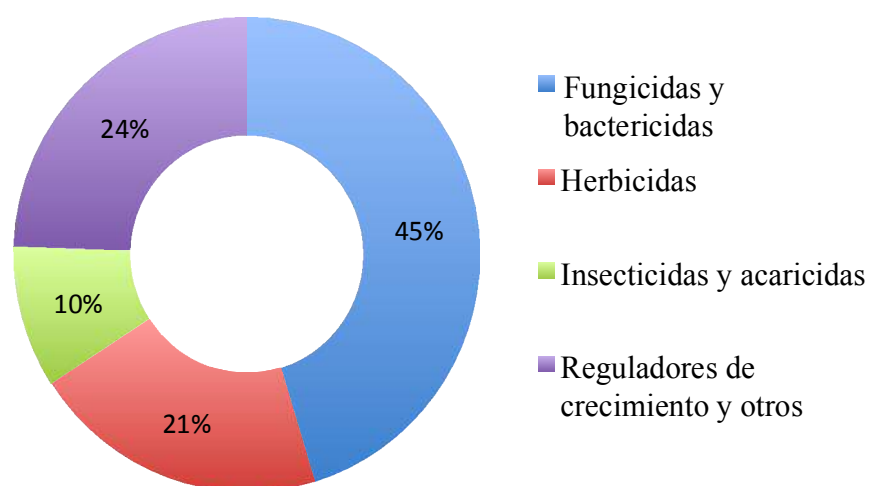


Figura 1. Principales grupos de sustancias químicas comercializadas en España (2013)

Dentro de estos grupos los fungicidas inorgánicos son los más utilizados, representando el 83.1 % de total de fungicidas y bactericidas y el 37.6 % del total de sustancias activas comercializadas. Dentro de los herbicidas la comercialización de las fenoxifitohormonas representa un 9.1 %. Los organofosforados con un 31.3 % son los más comercializados dentro de los insecticidas y acaricidas.

1.2. DINÁMICA DE PESTICIDAS EN SUELOS

La dinámica de los pesticidas en el suelo viene condicionada por multitud de procesos químicos, físicos y biológicos. Los mecanismos que influyen en la persistencia y evolución de los pesticidas en el suelo están representados en la Figura 2. Estos mecanismos pueden actuar solos o en combinación sobre los diferentes productos específicos y dependen de otras variables, como humedad, temperatura, contenido y naturaleza de la materia orgánica, tipo de arcilla, pH, intercambio iónico del suelo, así como de las propiedades químicas del pesticida aplicado (Morell y col, 1998).

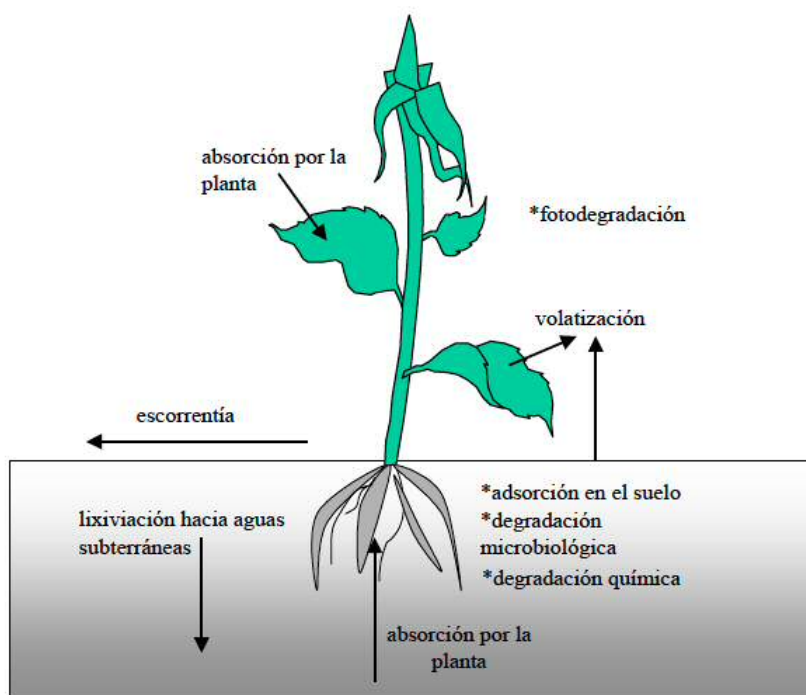


Figura 2. Procesos que afectan a los pesticidas en el medio ambiente (Malato Rodríguez y col., 2004)

1.2.1. Procesos: adsorción, lixiviación, degradación

El proceso de **adsorción** de pesticidas por el suelo se refiere a la interacción entre estos compuestos y las partículas del suelo. Este proceso tiene lugar en fase líquida, debido a que el pesticida es transportado disuelto en agua hasta la superficie de las partículas del suelo, las cuales se encuentran a su vez recubiertas por una película de agua.

Hay que dar una especial importancia al contenido en materia orgánica del suelo ya que es un factor decisivo en la adsorción. Cuando el contenido en materia orgánica es elevado, el coeficiente de correlación adsorción-materia orgánica también es elevado, teniendo lugar la adsorción principalmente en las superficies orgánicas y enmascarando el efecto de las superficies minerales en este proceso. Cuando este contenido es menor del 2%, comienza a manifestarse el efecto de los constituyentes inorgánicos en el proceso de adsorción (Real-Ojeda, 2009).

El proceso de adsorción influye directamente en el transporte, la volatilización y la degradación por microorganismos, afectando a este último en la medida en la que si un pesticida esta poco accesible a los microorganismos estos tardarán más tiempo en degradarlo (Barriuso y col., 1997).

La **lixiviación** es el movimiento vertical de los pesticidas a través del perfil del suelo originado por el agua de lluvia o del riego de los cultivos. El proceso de lixiviación es el principal responsable de la contaminación de las aguas subterráneas por pesticidas. Este proceso está asociado a dos fenómenos: Flujo preferencial que permite el movimiento rápido de las moléculas de pesticidas en el agua a través de los macroporos del suelo (favorece el movimiento vertical de los pesticidas en el suelo) y flujo matricial que permite el movimiento lento de moléculas de pesticidas en el agua a través de los microporos del suelo y por tanto mayor tiempo de contacto con las partículas del mismo, facilitando la difusión en el medio (proceso responsable del movimiento lateral del pesticida en el suelo) (Cohen y col., 1995; Whitford y col., 1995).

El proceso de **degradación** da lugar a la transformación del pesticida en compuestos más simples, como H_2O , CO_2 y NH_3 . Aunque este proceso pueda ser el

resultado de mecanismos abióticos, por ejemplo hidrólisis, fotólisis y degradación química, el principal instrumento de mineralización son los mecanismos bióticos: metabolismo y catabolismo microbiológico. La microbiota del suelo utiliza los pesticidas como fuente de carbono y otros nutrientes. Algunos productos químicos se descomponen muy rápidamente en el suelo, mientras que otros son muy persistentes y tardan mucho tiempo en descomponerse (Stephenson y Solomon, 1993).

La **fotodegradación** y la **hidrólisis** consisten en la degradación del pesticida como consecuencia de la acción de la luz o del agua, respectivamente, sobre la molécula. La fotodegradación depende directamente de la luz directa del sol, siendo posible en los primeros centímetros del suelo (Miller y col., 1983).

La **degradación química** es llevada a cabo a través de reacciones de hidrólisis y oxidación de los pesticidas sobre las superficies de arcillas, óxidos e iones metálicos, sustancias húmicas y materiales orgánicos, todos ellos presentes en el suelo, que actúan a modo de catalizador (Fernandes, 2006). El pH suele desempeñar un papel clave en dichas reacciones (Bending y col., 2006).

El proceso de **biodegradación** se basa en la susceptibilidad que tiene un pesticida a ser degradado por los microorganismos presentes en el suelo, condicionada a su vez por el grado de similitud de la estructura química del pesticida con la de los sustratos orgánicos naturales que son usados como fuente de alimento por las comunidades microbianas del suelo. Grandes moléculas orgánicas en forma de anillo o cadenas largas son degradadas mucho más lentamente que las cadenas más simples. En algunos casos, la exposición a un pesticida persistente durante un tiempo prolongado, ha inducido al desarrollo o proliferación de ciertas poblaciones microbianas capaces de degradar dichas estructuras, este fenómeno se conoce como capacidad de resistencia y resiliencia (Howell y col., 2014).

En el proceso de degradación la materia orgánica tiene un papel determinante ya que no sólo aumenta la adsorción de los pesticidas en el suelo, sino que es una fuente de nutrientes para los microorganismos del suelo. Debido a que el contenido en materia orgánica muestra un gradiente decreciente a medida que aumenta la profundidad del suelo, la velocidad de biodegradación una vez que el pesticida ha atravesado la capa superficial del suelo disminuye de forma proporcional (Rodríguez-Cruz y col., 2006).

1.2.2. Efecto de los pesticidas sobre las comunidades microbianas del suelo

La aplicación intensa y continuada de pesticidas sobre un suelo agrícola puede alterar tanto las propiedades del suelo como la estructura de las comunidades de los organismos que habitan en este, afectando negativamente a la calidad y productividad de los cultivos (Muñoz-Leoz y col., 2013). Dicho efecto negativo puede deberse a la alteración de las funciones naturales de la microbiota, como son la degradación de la materia orgánica de los restos del cultivo, la mejora del crecimiento de la planta, el reciclaje de nutrientes del suelo y la propia degradación de contaminantes y pesticidas (Jacobsen y col., 2014). A su vez, la alteración de las funciones de la microbiota del suelo debidas a interacciones directas con el pesticida estará determinada por la biodisponibilidad del mismo. Estas interacciones van a afectar a la abundancia y diversidad de microorganismos así como a la actividad de los mismos (Ahtiainen y col., 2003). El pesticida afecta a las comunidades microbianas del suelo mediante un efecto doble, por un lado mediante la eliminación de aquellas especies sensibles a la toxicidad de los compuestos aplicados y por otro por una selección positiva de aquellas que toleran la toxicidad y que pueden usar el pesticida como fuente de carbono para crecer (Howell y col., 2014). A su vez, la presencia del pesticida va a afectar al metabolismo de los microorganismos que activarán rutas de degradación del mismo, lo que permite su uso como indicadores biológicos (Howell y col., 2014).

Una vez que el pesticida entra en contacto con el suelo se ve afectado por transformaciones biológicas o fisicoquímicas, pero será la **degradación del pesticida, debido al aumento de poblaciones microbianas** que utilicen este como fuente de energía, la principal ruta de degradación de este (Bending y col., 2006). Baelum y col. (2008) observaron que al aplicar repetidamente el herbicida MCPA se producía un incremento en poblaciones capaces de degradar el compuesto. Sopeña y Bending (2013) y Howell y col. (2014) estudiaron el efecto de las comunidades de hongos en la disipación del azoxystrobin, observando en ambos casos que la disipación del pesticida es más lenta cuando se aplican altas concentraciones de pesticida. Además en el ensayo realizado por Sopeña y Bending (2013) se observaron variaciones en las poblaciones de hongos presentes en el suelo tras la aplicación del pesticida.

La **biodisponibilidad** de un pesticida es uno de los parámetros más determinantes a la hora del estudio de la influencia de un pesticida sobre la microbiota

del suelo, debido a que si un pesticida está biodisponible estará expuesto a los organismos, mientras que si tiene una baja biodisponibilidad el acceso a este se hará mucho más difícil (Jacobsen y col., 2014).

La complejidad del estudio de la diversidad microbiana ya fue reflejada en 1934 por el microbiólogo holandés Lourens Baas Becking en su hipótesis “*Everything is everywhere, but the environment selects*” (Baas Becking, 1934), reflejando la rápida adaptación de las comunidades al medio y la dificultad de distinguir los factores que producen estos cambios. La **capacidad de resistencia y/o adaptación** a la exposición de fungicidas ha sido estudiada por Howell y col. (2014), observando que la aplicación del fungicida azoxistrobin afecta solamente a ciertas comunidades de hongos y nematodos. Widenfalk y col. (2008) realizaron un estudio sobre los posibles efectos tóxicos de los pesticidas sobre los microorganismos. En este estudio no se observaron cambios significativos en la estructura de las comunidades bajo la aplicación del insecticida pirimicarb, atribuyendo estos resultados a que la actividad bacteriana muestra una respuesta instantánea a la exposición del pesticida, pero también una recuperación, debido a la adaptación fisiológica (hacia especies más resistentes).

La respiración, la biomasa y la nitrificación son algunos de los **indicadores biológicos** más ampliamente utilizados para medir la calidad del suelo y por consiguiente las alteraciones en las comunidades. La medición de las actividades enzimáticas también pueden usarse para describir los cambios en la diversidad microbiana. Floch y col. (2011) han demostrado que la actividad de la fenol oxidasa es un buen indicador para reflejar los efectos de los pesticidas a corto plazo, mientras que la β -glucosidasa puede usarse para evaluar la resiliencia de los microorganismos.

1.3. PROBLEMÁTICA DEL USO DE PESTICIDAS: CONTAMINACIÓN DE SUELOS Y AGUAS

1.3.1. Legislación sobre la contaminación de suelos y aguas. Fuentes de contaminación

El suelo se considera un componente esencial para el desarrollo de la vida, pero es vulnerable, además posee una difícil y larga recuperación, por lo que se considera un recurso natural no renovable (www.fao.org/soils-2015).

El suelo desarrolla importantes funciones ambientales; es el soporte de alimento para las plantas, almacena nutrientes, posee y alberga materia orgánica proveniente de restos animales y vegetales y es el hábitat de diversos organismos que transforman la materia orgánica presente en él (Arroyave y col., 2009). También actúa como amortiguador natural, controlando el transporte de elementos y sustancias químicas a la atmósfera y al agua. Por tanto, se dice que el mantenimiento de las funciones ecológicas del suelo es responsabilidad del hombre (Kabata-Pendias y col., 1992).

En cuanto a legislación ambiental no es hasta 1972 cuando la Unión Europea redacta la “Carta Europea de Suelos” declarando que este debe ser protegido contra la erosión, la contaminación y los daños causados por el desarrollo urbano y las prácticas agrícolas. Razón por la que los gobiernos deben impulsar medidas específicas para salvaguardar este recurso (Solano, 2005).

En la Cumbre de Río (1992) se reconoció la importancia de la protección de los suelos y de sus usos potenciales en el contexto de un desarrollo sostenible, en particular contra la contaminación de origen antropogénico. En este mismo año, la Convención Marco sobre Cambio Climático (CCC) planteó los problemas de la degradación del suelo y los cambios en los usos del mismo que podían agravar el efecto invernadero.

En 2001, la Comisión de la Comunidad Europea advirtió del peligro que la erosión y el declive de la fertilidad del suelo representan para el desarrollo sostenible. Debido a lo anterior, numerosos países empezaron a considerar en sus políticas ambientales el recurso suelo, creando una normativa ambiental para la gestión, conservación y protección del mismo.

España redacta en 1986 la Ley Básica de Residuos Tóxicos y Peligrosos, apoyando con esta normativa la recuperación de los espacios contaminados con sustancias peligrosas. En 1998, se estableció una legislación básica en materia de protección de suelo frente a la contaminación, promulgando por primera vez una normativa que permitía proteger los suelos contra la contaminación.

En 2005 se establece una metodología normalizada para caracterizar suelos contaminados (RD 9/2005, de 14 de enero). Definiendo **suelo contaminado**, como aquel cuyas características han sido alteradas negativamente por la presencia de componentes químicos de carácter peligroso de origen humano, en concentración tal que comporte un riesgo inaceptable para la salud humana o el medio ambiente (RD 9/2005, de 14 de enero).

Haciendo uso de la definición del suelo que da el decreto anterior: “**Suelo**: la capa superior de la corteza terrestre, situada entre el lecho rocoso y la superficie, compuesto por partículas minerales, materia orgánica, agua, aire y organismos vivos y que constituye la interfaz entre la tierra, el aire y el agua, lo que le confiere capacidad de desempeñar tanto funciones naturales como de uso. No tendrán tal consideración aquellos permanentemente cubiertos por una lámina de agua superficial”. Esta definición no se limita al denominado suelo edáfico sino que también incluye el material que se encuentra en la subsuperficie a través de la cual los contaminantes pueden llegar hasta las **aguas subterráneas**. Desde un punto de vista técnico, no se puede hablar de contaminación de un suelo sin considerar la posible contaminación de las aguas, en especial de las subterráneas (Figura 3). De hecho, la Ley de las Aguas (RD 1/2001, de 20 de julio) hace referencia a la protección de los acuíferos como límite físico de las aguas subterráneas.

Se entiende por **agua subterránea** aquella almacenada por debajo de la superficie del suelo, en los espacios porosos del suelo, en las grietas y fracturas de las formaciones rocosas y por **agua superficial**, aquella que se encuentra en la superficie de la tierra, la cual por definición puede abarcar desde lagunas o pozos efímeros (estacionales) hasta agua permanente como canales, ríos, embalses, estuarios y mares (Carter, 2000).

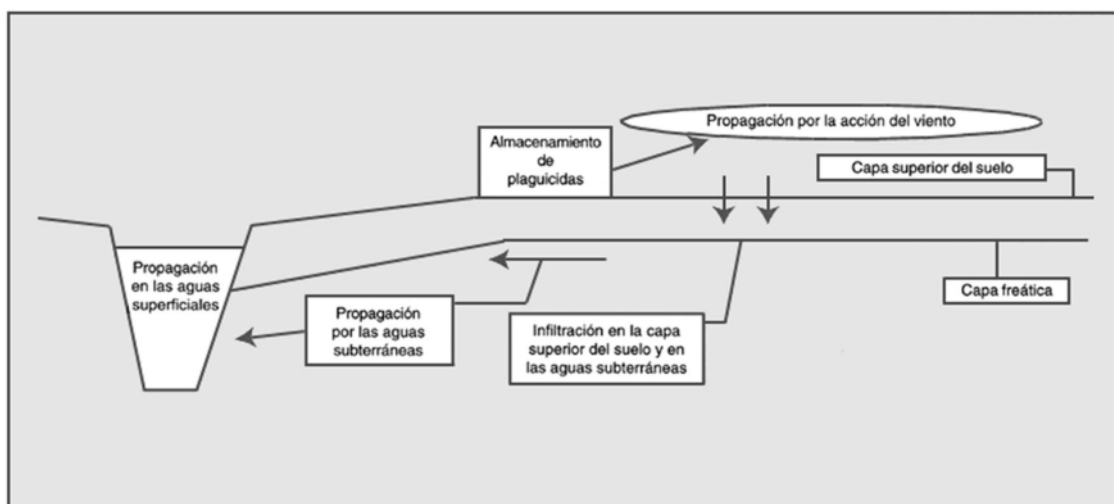


Figura 3. Dispersión de los contaminantes al medio ambiente (FAO, 2000)

Los pesticidas pueden alcanzar las aguas mediante los procesos de movilidad previamente explicados en el apartado 1.2.1. Se ha demostrado que la entrada de pesticidas al agua se produce por numerosas vías.

La **contaminación de las aguas por pesticidas** puede proceder de fuentes no localizadas o **contaminación difusa**, o de **fuentes localizadas o puntuales**. La contaminación difusa implica la presencia de contaminantes a baja concentración en un área extensa. Los pesticidas aplicados en los campos de cultivo, alcanzan en un primer momento la capa del suelo microbiológicamente activa, donde tienen lugar los procesos de disipación o degradación. La sustancia activa y/o sus metabolitos se moverán hacia las siguientes capas de suelo adsorbidas a partículas del suelo o en solución, para posteriormente entrar en el agua a través de los sistemas de drenaje, escorrentía o flujo preferencial. La repercusión de la contaminación difusa dependerá de la magnitud de los procesos de adsorción, movilidad y degradación que experimentan los pesticidas en la zona no saturada del suelo.

La contaminación puntual de las aguas por pesticidas puede estar originada por diversas fuentes (fugas desde los equipos de aplicación, derrames o disposición incorrecta de los residuos y aguas de lavado de los utensilios o maquinaria de aplicación). La contaminación puntual implica la presencia de concentraciones elevadas de contaminante en un área más limitada (Tabla 3).

Tabla 3. Fuentes de entrada de pesticidas y tipos de aguas afectadas (Carter, 2000)

| Fuente de entrada | Ruta/causa de entrada | Tipo de agua afectada |
|--------------------------|--|------------------------------------|
| DIFUSA | Pulverización | Zanjas, arroyos, estanques, ríos |
| | Volatilización y precipitación | Zanjas, arroyos, estanques, ríos |
| | Escorrentía y flujo superficial, | Zanjas, arroyos, estanques, ríos |
| | Lixiviación | Aguas subterráneas |
| | Flujo | Zanjas, arroyos, estanques |
| | Drenaje | Zanjas, arroyos, estanques |
| | Infiltración del caudal | Aguas subterráneas y superficiales |
| PUNTUAL | Llenado de tanques | Aguas subterráneas y superficiales |
| | Derrames | Aguas subterráneas y superficiales |
| | Equipamiento defectuoso | Aguas subterráneas y superficiales |
| | Lavado y eliminación de residuos | Aguas subterráneas y superficiales |
| | Sumideros, pozos de adsorción y drenajes | Aguas subterráneas y superficiales |
| | Entradas directas | Zanjas, arroyos, estanques, ríos |
| | Descargas | Arroyos y ríos |

La concentración máxima de pesticidas permitida en aguas está regulada por una serie de directivas que intentan evitar la contaminación de los ecosistemas acuáticos, evitando así el riesgo para la salud humana. La Directiva Europea (Directiva 80/778/EEC) sobre la potabilidad del agua y la Directiva revisada 98/83/EEC, sin que se estableciera cambio alguno en los parámetros para los pesticidas, establecen que el nivel individual de un pesticida en agua potable no puede exceder la concentración de $0.1 \mu\text{g L}^{-1}$ y la concentración total de pesticidas en el agua no puede exceder de $0.5 \mu\text{g L}^{-1}$. Estos valores también son válidos para el agua superficial cuando se utiliza como agua de bebida, de conformidad con la directiva 75/440/EEC (Carter, 2000).

El aumento del consumo de **pesticidas** está agravando la contaminación de las aguas subterráneas. Un pesticida ideal sería selectivo y no persistente, sin embargo la mayoría de los pesticidas inducen efectos adversos en los organismos y tienden a acumularse en los ecosistemas. Por ello, la Directiva sobre el registro de productos fitosanitarios (91/414/EEC) intenta evitar el impacto de estos compuestos en los organismos a los que no van destinados, tanto en ecosistemas acuáticos como en terrestres. Los efectos de los pesticidas en la **calidad del agua** están asociados a los siguientes componentes:

- ✓ Ingredientes activos en la formulación de los pesticidas.
- ✓ Contaminantes que existen como impurezas junto con el ingrediente activo.
- ✓ Aditivos que se mezclan con el ingrediente activo (humectantes, diluyentes o solventes, aprestos, adhesivos, soluciones reguladoras, conservantes y emulsionantes).
- ✓ Productos de degradación o metabolitos que se forman durante la degradación química, microbiana o fotoquímica del ingrediente activo.

1.3.2. Monitorización de pesticidas en suelos y aguas

Los estudios de **monitorización** de contaminantes surgen con el fin de realizar informes medioambientales de las emisiones de sustancias potencialmente contaminantes o para comprobar el cumplimiento de la legislación. Hay varias consideraciones a tener en cuenta a la hora de realizar un estudio de monitorización como son el momento y el protocolo seguido para la toma de muestra y los procedimientos seguidos hasta su determinación analítica. La obtención de los datos de monitorización sigue varios pasos consecutivos que necesitan realizarse de acuerdo con normas o protocolos específicos para asegurar unos resultados de buena calidad. Estos pasos pueden englobarse en el muestreo, almacenaje, transporte y preservación de la muestra, tratamiento de la muestra, análisis, procesamiento de datos y elaboración del informe (MAGRAMA, 2003). Para la monitorización de pesticidas se requiere desarrollar métodos de análisis con niveles de detección que tengan significado para la protección de la salud humana y del ecosistema y distinguir entre los contaminantes que aparecen como resultado de usos anteriores y los que se están utilizando en el presente (FAO, 2015).

El incremento de la detección de pesticidas en las **aguas** puede ser atribuido por una parte al desarrollo de una generación de pesticidas con una mayor capacidad de degradación y lixiviación, unido a un aumento de su utilización y por otra parte, al desarrollo de métodos de análisis con mayor sensibilidad que permiten determinar concentraciones cada vez más bajas.

El Instituto Nacional de Salud Pública y Protección Ambiental de los Países Bajos (RIVM, 1992) notificó que “el agua subterránea está amenazada por los pesticidas en todos los estados europeos”. En países europeos como Francia (Talbot y col., 2015), Reino Unido (Jürgens y col., 2015), Portugal (Cruzeiro y col., 2015), Grecia (Papadakis y col., 2015) o Irlanda (McManus y col., 2014) entre otros, también se ha detectado la presencia de pesticidas en aguas superficiales y subterráneas. Haarstad y col. (2007) realizaron, entre los años 1995-2004, un estudio de monitorización en Noruega. Los resultados indicaron que el agua subterránea cercana a explotaciones agrícolas es especialmente vulnerable a la contaminación por pesticidas. Malguerra y col. (2011) desarrollaron un análisis estadístico estudiando diversas variables para predecir la vulnerabilidad de la contaminación por pesticidas de las aguas danesas. Los resultados mostraron una mayor vulnerabilidad de las aguas de los pozos con menor profundidad.

En España no existe un seguimiento regular sistemático de los pesticidas en las masas de agua, pero varios estudios señalan su presencia cada vez más abundante en las aguas subterráneas, sobre todo como consecuencia directa de las actividades agrícolas (OECD, 2004; Hildebrant y col., 2008; Herrero-Hernández y col., 2013). El regadío ha provocado la contaminación de acuíferos, en algunos casos por encima de los niveles máximos permitidos en la legislación europea para el agua potable, como ocurre en España en las cuencas del Ebro, y Júcar (Hildebrant y col., 2009; Navarro y col., 2010; Belenguer y col., 2014). Además, todavía se siguen detectando en los suelos, el agua, los alimentos y las personas algunos pesticidas organoclorados (OCPs) cuyo uso se había limitado o prohibido a finales de la década de los 70 y mediados de los 80, por ejemplo, DDT y sus metabolitos (σ DDs), aldrín, endrín, isodrín, γ -HCH y HCB, debido a su persistencia en el medio ambiente (Carreño y col., 2007; Carro, 2014).

En los últimos años en España se han realizado estudios de monitorización que muestran la presencia de pesticidas y otras sustancias contaminantes en ríos y aguas

subterráneas. Ccanccapa y col. (2016) monitorizaron durante dos años la presencia de 50 pesticidas en agua y sedimentos de los ríos Turia y Júcar (Comunidad Valenciana), demostrando la contribución tanto de las actividades agrícolas como urbanas a la contaminación por pesticidas. Los resultados mostraron la presencia en altas concentraciones de cloropirifos, imazalil, tiabendazol, tolclofos metil, etion y carbofurano. Las áreas con mayor contaminación se encontraron en las cabeceras de los ríos, y a su vez, la variación temporal indicó una clara relación de la hidrología del río con un mayor caudal y se encontró mayor presencia de pesticidas pero a concentraciones bajas. Por el contrario, a bajos caudales la concentración de los pesticidas detectados aumentó. Robles-Molina y col. (2015) llevaron a cabo la monitorización de 373 compuestos (pesticidas, PAHs, nitrosaminas, compuestos farmacéuticos, etc.) en aguas superficiales de la provincia de Jaén. Las sustancias más frecuentemente detectadas fueron los pesticidas terbutilazina, oxifluorfen, desetil terbutilazina y difenilamina.

En la monitorización de 22 pesticidas en 112 muestras de aguas subterráneas realizada por Köck-Schulmeyer y col. (2014) a lo largo de cuatro años, se detectó la presencia a altas concentraciones de simazina, atrazina y diurón. Este estudio también mostró una relación entre la frecuencia de detección y el parámetro GUS (Gustafson, 1989). El índice GUS integra los dos factores más influyentes en la lixiviación de los pesticidas: su persistencia en el suelo expresada mediante la vida media, $t_{1/2}$ y su adsorción por el suelo, expresada por el coeficiente de adsorción, Koc.

El estudio realizado en el río Llobregat (Masiá y col., 2015b), con el fin de monitorizar la contaminación en la ribera del río, reveló la presencia de pesticidas de las familias de los benzimidazoles, organofosforados y ureas en agua, sin embargo su presencia no fue tan alta en las muestras de sedimentos. Estos estudios pueden ser de gran utilidad para controlar la afluencia de contaminantes y confirmar la presencia de compuestos tóxicos y sus productos de degradación.

El desarrollo de nuevas técnicas analíticas y/o métodos de extracción han permitido la evaluación de residuos de pesticidas en suelos en concentraciones bajas. Los métodos convencionales de extracción de pesticidas desde el suelo incluían técnicas como la sonicación con disolventes orgánicos (Brunete y col., 2003), extracción líquido – sólido (Prakash y col., 2004) o la extracción Soxhlet (Arora y col., 2008).

Hasta la fecha no son muchos las publicaciones sobre determinación de residuos de pesticidas en suelos de España. Belmonte-Vega y col. (2005) analizaron la presencia de 32 pesticidas en suelos y aguas de Andalucía, mediante cromatografía líquida (LC-MS) siendo los herbicidas simazina, diuron y terbutilazina los compuestos detectados en mayor número de muestras. Bermúdez-Couso y col. (2007) analizaron sedimentos de ríos en la provincia de Ourense, cercanos a zonas de viñedo mediante extracción sólido-líquido. Los pesticidas encontrados (procimidona, ciprodinil, fludioxonil, metalaxil y penconazol) se detectaron en cantidades muy bajas indicando que el suelo no debería favorecer el transporte de estos compuestos hacia el agua. Hildebrandt y col. (2009) llevaron a cabo una monitorización durante tres años en suelos cercanos al río Ebro, donde se estudiaron 22 pesticidas organoclorados y algunos de sus productos de degradación. El 53% de los suelos analizados contenían entre 0.13 y 58.17 $\mu\text{g kg}^{-1}$ de 4,4'-DDT, mientras que el 88% contenían 4,4'-DDE en el mismo rango de concentraciones, indicando una disipación más rápida de DDT.

A nivel mundial se han realizado numerosos estudios sobre evaluación de contaminantes en suelos, sobre todo en países emergentes como China e India. Yu y col. (2013) encontraron altos niveles de pesticidas organoclorados (OPCs) en el delta de río de las Perlas (China). Shi y col. (2011) detectaron en suelos suburbanos de la zona de Shenyang (China) contaminantes persistentes de la familia del DDT y hexaclorociclohexanos (HCHs). Los mismos compuestos fueron detectados en suelos de Nagaon y Dibrugarh (India) por Mishra y col. (2012) y en Pakistán por Ahad y col. (2010). Manz y col. (2001) estudiaron la degradación de contaminantes persistentes, detectados en Alemania, con el fin de elucidar si los residuos que habían detectado en muestras de suelo provenían de una contaminación actual o pertenecían a una contaminación anterior. Kalogridi y col. (2014) desarrollaron un método para monitorizar 253 pesticidas en sedimentos en tres lagos griegos, observando que los pesticidas del grupo de las triazinas, triazoles y organofosfatos eran los grupos predominantes.

1.3.3. Evaluación de la contaminación por pesticidas en las zonas de viñedo de La Rioja

En la Comunidad de La Rioja las necesidades de agua para el abastecimiento de poblaciones (40 hm³/año) se atienden mediante aguas superficiales en un 59% (que abastecen al 10% de los municipios) y mediante aguas subterráneas en un 28% (que abastecen al 66% de los núcleos de población, con más de 80000 habitantes) que proceden en su mayor parte de los acuíferos del Ebro (aluvial del Ebro-Cenicero-Lodosa (22%), aluvial del Ebro-Lodosa-Tudela (27%) y aluvial del Oja (15%)). La calidad de estas aguas superficiales y subterráneas se considera aceptable de acuerdo con el Plan Director de abastecimiento de agua del Gobierno de esta Comunidad 2002-2015 (www.larioja.org), excepto en algunos sectores de los acuíferos aluviales donde se han identificado altas concentraciones de nitratos. Sin embargo, son muy escasos los datos respecto a las concentraciones de pesticidas que pudieran existir en las mismas. Solamente se han publicado algunos resultados de investigación referidos principalmente a compuestos triazínicos (Hildebrandt y col., 2007; Villaverde y col., 2008), a pesar de que más de 156000 hectáreas están dedicadas a la agricultura y el consumo medio de pesticidas (fungicidas, herbicidas e insecticidas) se estima en 11.78 kg ha⁻¹ en La Rioja frente a los 6.78 kg ha⁻¹ de media total en España (Estadística Agraria Regional de La Rioja, 2012. www.larioja.org).

Estos datos pusieron de manifiesto el interés de llevar a cabo una monitorización de los pesticidas más utilizados, tanto en suelos como en aguas superficiales y subterráneas, en zonas de cultivo en régimen de secano y regadío de La Rioja para obtener un diagnóstico ambiental relativo a la contaminación por estos compuestos. Es de especial interés el posible aporte de pesticidas al agua procedente de la actividad vitivinícola de esta región, debido a las grandes cantidades de pesticidas aplicados. La superficie de viñedo productivo en el año 2014 fue de 61645 ha y su interés se basa en la elevada actividad económica que proporciona a la misma debido a la gran cantidad de vino que permite elaborar (281 millones de litros en el año 2014) (Consejo regulador D.O. Calificada Rioja, 2015. www.riojawine.com).

1.4. RESIDUOS ORGÁNICOS

1.4.1. Antecedentes

El aumento de la superficie destinada a la producción de cultivos a nivel mundial aumentó un 12% de 1960 a 2010, mientras que el aumento de la productividad agrícola mundial en el mismo periodo fue de 150% - 200% (FAO, 2010). Con estos datos es evidente que el aumento de la productividad no se debe solamente al aumento de superficie cultivada si no que hay otros factores que han propiciado este incremento, como es la utilización de recursos hídricos, el desarrollo tecnológico de maquinaria agrícola, el uso de semillas modificadas genéticamente y el uso extensivo de pesticidas, explicado anteriormente. Estas actividades antropogénicas están acelerando la degradación del suelo a través de la alteración de las propiedades físicas, químicas y biológicas de este. La pérdida de **materia orgánica** está alterando la concentración de nutrientes presentes en el suelo, y disminuyendo la capacidad de retención de agua debido a la menor porosidad y capacidad de infiltración. La actividad microbiana del suelo también está sufriendo efectos negativos. Además la degradación del suelo se ve agravada por otro problema medioambiental como es la contaminación del suelo por sustancias tóxicas (metales pesados, pesticidas, productos farmacéuticos y cosméticos, etc.) y la posterior infiltración de estos al agua (FAO, 2015).

1.4.2. Uso de residuos orgánicos en la agricultura

La adicción de residuos orgánicos como enmiendas en agricultura ha sido una práctica generalizada debido a que el aumento de materia orgánica puede mejorar la calidad del cultivo mediante el aporte de nutrientes, el incremento de la retención de agua, la variación del pH, el aumento de la aireación, etc. (Teca, 2015). Entre los residuos orgánicos potencialmente aprovechables en agricultura como enmiendas y/o fertilizantes se encuentran los residuos urbanos tales como lodos de depuradora, los residuos derivados de la producción agrícola y de la industria agroalimentaria. Entre los residuos derivados de la producción agrícola se incluyen restos de cosechas, podas y purines. Dentro del grupo de desechos de la industria agroalimentaria cabe destacar los residuos generados en el cultivo del champiñón y setas comestibles.

El uso de estos residuos orgánicos se ha potenciado también en los últimos años como adsorbentes de contaminantes (Dolaptsoglou y col., 2007; Rojas y col., 2013; Rodríguez-Salgado y col., 2014; Vithange y col., 2015). En la Figura 4 se muestra el aumento de publicaciones científicas incluidas en el Science Citation Index (SCI) en las que se cita el uso de estos materiales residuales como adsorbentes de contaminantes.

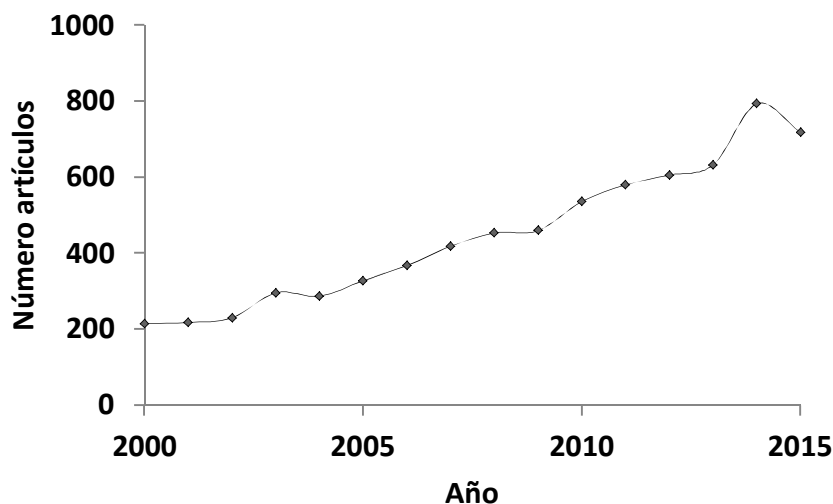


Figura 4. Tendencia de artículos publicados en revistas SCI sobre residuos orgánicos (Scopus, octubre 2015)

1.4.3. Efecto de los residuos orgánicos en las comunidades microbianas del suelo

Los residuos orgánicos utilizados como enmiendas orgánicas en el suelo pueden inducir cambios en las comunidades microbianas del suelo ya sea mediante la incorporación de nuevas especies o mediante la alteración de las existentes. Las enmiendas orgánicas pueden modificar la abundancia, actividad, funcionamiento y estructura o diversidad de las comunidades microbianas del suelo, tal y como se ha puesto de manifiesto en estudios llevados a cabo en los últimos años (Pérez-Piqueres y col., 2006).

En general, la adicción de diferentes tipos de enmiendas al suelo está relacionada con el aumento de la **biomasa microbiana** (Bonilla y col., 2012). Este incremento está directamente ligado al tipo de enmienda aplicada. Saison y col. (2006) mostraron el

aumento de las bacterias heterotróficas y Bulluck y col. (2002) observaron un incremento de *Pseudomonas spp.* fluorescentes cuando se añadía al suelo compost comercial de origen animal o vegetal.

Las enmiendas orgánicas no influyen solamente en la abundancia de las poblaciones, también lo hacen sobre la **diversidad en las comunidades** (Pérez-Piqueres y col., 2006). Mientras que el efecto de las enmiendas orgánicas sobre la biomasa ha sido estudiado con detalle, el estudio del impacto sobre la estructura de las comunidades microbianas es aún incipiente debido al desarrollo de nuevas técnicas de biología molecular. Las técnicas de secuenciación han permitido examinar con detalle los cambios producidos en la estructura de las comunidades en función de las enmiendas aplicadas (Hartmann y col., 2015). Los métodos moleculares basados en la secuenciación de ácidos nucleicos han sido la clave para desarrollar los estudios de la diversidad genética y funcional en el suelo. Como consecuencia del desarrollo no solo de las tecnologías de secuenciación (“next-generation” y “high-throughput”) sino también de las herramientas para el análisis bioinformático, se ha ampliado el conocimiento de la diversidad de los ecosistemas (Figura 5). La secuenciación de ADN nos permite conocer al mismo tiempo la filogenia y los genes funcionales de las comunidades microbianas.

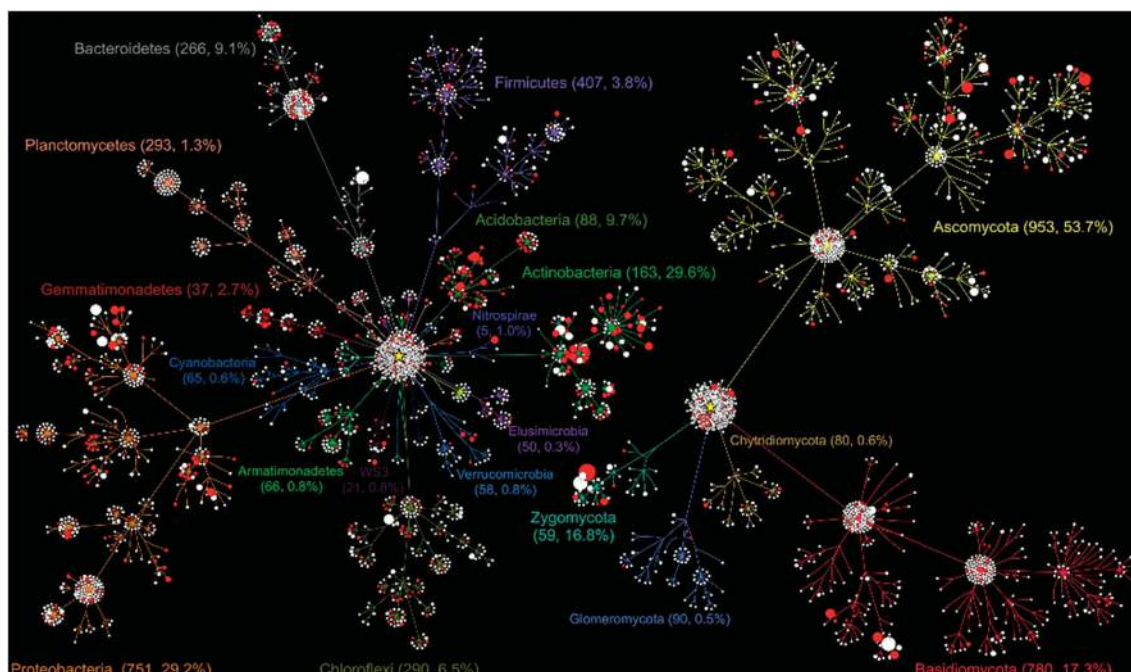


Figura 5. Dendrogramas taxonómicos de las comunidades bacterianas y de hongos detectadas en una muestra de suelo (Hartmann y col., 2015)

Como se ha indicado, la incorporación de materia orgánica al suelo puede jugar un papel fundamental en la degradación, transporte e inmovilización de muchos contaminantes. La aplicación de enmiendas puede afectar a la biodegradación de pesticidas mediante:

- ✓ Estimulación de la microflora endógena o introduciendo microorganismos exógenos al suelo
- ✓ Alterando la movilidad del pesticida y por tanto, las probabilidades de contacto con los microorganismos capaces de degradarlo
- ✓ Modificando las características del suelo que puedan afectar a la actividad microbiana

Sin embargo, existen estudios (Frossard y col., 2012), en los que los cambios filogenéticos no están relacionados con las propiedades del suelo. De hecho se ha demostrado el efecto que tienen las enmiendas orgánicas sobre las propiedades del suelo sin alterar la estructura microbiana del mismo (Crecchio y col., 2012). Por tanto, el estudio de la filogenia del suelo no es suficiente para entender el funcionamiento de las comunidades microbianas. La **actividad microbiana** puede ser analizada mediante **respiración del suelo** o ciclos biogeoquímicos de carbono o nitrógeno. También, la respuesta enzimática del suelo está relacionada con el contenido en materia orgánica del mismo y la aplicación de residuos orgánicos puede modificarla, por ello las **actividades enzimáticas** como la fosfatasa, ureasa, deshidrogenasa y β -glucosidasa son indicadoras de la actividad microbiana del suelo y pueden verse alteradas en suelos enmendados con residuos orgánicos tal y como se ha puesto de manifiesto en distintos estudios (García-Ruiz y col., 2008).

1.4.4. Residuo postcultivo de champiñón: Generación y problemática

La producción de 1 kg de champiñón genera 5 kg de residuo orgánico llamado residuo postcultivo de champiñón (en inglés, spent mushroom substrate, SMS). Este residuo está compuesto por los materiales del compost inicial (paja, serrín, estiércol y yeso) utilizado para la producción de los hongos y aunque contengan nutrientes resulta más económico remplazar este sustrato para el siguiente cultivo (Figura 6). El SMS contiene un alto grado de materia orgánica y nutrientes lo que lo hace idóneo para su

uso como enmienda del suelo (Rodríguez-Cruz y col., 2012; Marín-Benito y col., 2013, 2014).



Figura 6. Producción de champiñón y residuo postcultivo de champiñón.

China es el mayor productor mundial de este residuo generando al año 20 mt. Holanda, Francia, Estados Unidos, Irlanda y España son también grandes productores de este residuo (FAOSTAT, 2015; Phan y col., 2012).

En el caso de España, la Comunidad de la Rioja produce más de 170000 t anuales de residuos postcultivo de champiñón, ya que el cultivo de hongos es la segunda actividad económica más importante en esta comunidad con una producción anual de 69300 toneladas champiñón y setas en 2013 (MAGRAMA, 2015).

La utilización como adsorbente del SMS contribuiría a su revalorización y al mismo tiempo a resolver el problema ambiental que provoca su acumulación en vertederos ya que según el artículo 5 del Real Decreto 1481/2001 de 27 de diciembre la Administración General del Estado y las Comunidades Autónomas deberán elaborar un programa de actuación para reducir los residuos orgánicos mediante el reciclaje y compostaje, evitando así los impactos negativos que provocan la acumulación de estos residuos en el medio ambiente (PNIR 2008-2015, Anexo 14).

1.4.5. Uso del residuo postcultivo del champiñón como instrumento para prevenir la contaminación de suelos o recuperar suelos contaminados

Debido al aumento de la contaminación por pesticidas, explicada anteriormente, se han establecido Directivas Europeas para desarrollar medidas específicas para

prevenir la contaminación de los suelos y limitar la entrada de pesticidas al agua. (Directivas 76/464/CEE, 2000/60/CE, 2006/118/CE y 2008/105/CE). Para proteger los acuíferos de la contaminación se están estudiando métodos fisicoquímicos de inmovilización de estos compuestos en el suelo para favorecer la adsorción y degradación de los contaminantes y disminuir los procesos de transferencia a otros medios, como la movilidad o la volatilización.

El SMS podría ser utilizado como material adsorbente para estos fines de prevención y/o control de la contaminación de suelos y aguas por pesticidas. Debido a que un gran número de pesticidas son compuestos orgánicos no-iónicos, con diferentes grados de hidrofobicidad y solubilidad en agua, estos son adsorbidos por otros compuestos orgánicos hidrofóbicos o por materiales orgánicos ricos en estos compuestos tales como el SMS.

Los pesticidas adsorbidos-inmovilizados pueden ser degradados al aumentar su tiempo de residencia en el suelo (cuando estos están biodisponibles para los microorganismos presentes en el suelo). También pueden quedar retenidos por la materia orgánica del residuo, experimentando una serie de procesos denominados de envejecimiento (redistribución desde lugares de adsorción débiles a lugares de adsorción más fuertes, adsorción química o formación de enlaces covalentes entre estos compuestos y la materia orgánica del residuo) que disminuyen su biodisponibilidad para ser biodegradados.

Gao y col. (2015) estudiaron el uso del SMS en “biobeds” o sistemas utilizados para retener y degradar pesticidas como un posible sustituto de la turba. Los pesticidas clorotalonil e imidacloprid presentaron un aumento en la velocidad de degradación debido al aumento de la actividad microbiana como consecuencia del aporte de una nueva fuente de carbono orgánico. Karanasios y col. (2010) también usaron SMS como material alternativo en biomezclas, observando que el aporte de este residuo incrementaba la adsorción de terbutilazina y metribuzina en comparación con los “biobeds” convencionales.

El SMS ha sido también aplicado para la degradación de los insecticidas carbaril, carbofuran y aldicarb (Regan, 1994) y del fungicida clorotalonil (Córdoba

Juárez y col., 2011) en el suelo y en agua (proveniente de la limpieza de los tanques de lavado).

La biorremediación es una tecnología basada en la utilización de los microorganismos y su potencial degradador para eliminar los contaminantes del medio, mediante su transformación en productos inocuos como el CO₂ y el H₂O. La búsqueda de nuevas fuentes enzimáticas provenientes de residuos ha llevado a algunos autores a estudiar el SMS como posible fuente de enzimas lignocelulósicas como lacasas, xilanasas, lignina peroxidasas, celulasas y hemicelulasas. Son numerosos los estudios que proponen el uso de SMS como una fuente disponible y barata de enzimas para biorremediación ambiental (Phan y col., 2012; González-Matute y col., 2012). García-Delgado y col. (2015a,b) presentaron dos alternativas de biorremediación usando SMS. En principio usaron el SMS para bioestimular la microbiota existente del suelo para activar la degradación de hidrocarburos aromáticos policíclicos, y posteriormente estudiaron el SMS como portador de hongos capaces de degradar por si solos estos contaminantes. Purnomo y col. (2010, 2011) han propuesto mecanismos de degradación del DDT usando SMS como técnica de biorremediación.

1.4.6. Influencia del sustrato postcultivo de hongos aplicado al suelo en el comportamiento de pesticidas

Estudios llevados a cabo en laboratorio y/o campo previamente a la realización de este trabajo han puesto de manifiesto la influencia de la aplicación de distintos tipos de SMS en el comportamiento de fungicidas aplicados en suelos de viñedo. Los resultados de estos trabajos sirvieron de base para plantear el uso del SMS en tecnologías fisicoquímicas para la prevención y/o control de la contaminación de suelos y aguas por pesticidas, el cual es uno de los objetivos del trabajo presente.

El estudio de la adsorción de los fungicidas metalaxil, penconazol, pirimetanil e iprovalicarb por suelos enmendados con SMS puso de manifiesto el aumento de la adsorción de estos pesticidas respecto al suelo no enmendado (Marín-Benito y col., 2012). Además, en todos los suelos enmendados se observó un descenso de la variabilidad de los coeficientes de adsorción (K_{OC}) de los fungicidas, debido a la influencia del carbono orgánico del residuo independientemente de otras propiedades fisicoquímicas de los suelos. Este estudio también reveló la influencia de la

composición del SMS y de la dosis aplicada en el proceso de desorción de los pesticidas desde los suelos enmendados.

Marín-Benito y col. (2009 a, b) llevaron a cabo un estudio de lixiviación de metalaxil y penconazol en suelos de viñedo enmendados con SMS, observando que la movilidad del metalaxil fue mayor que la del penconazol debido a su menor adsorción por el suelo. Esta menor retención del metalaxil facilita su degradación y explica los bajos porcentajes de compuesto total encontrado en las columnas después de la incubación. En este estudio se observó también que la adición de la enmienda daba lugar a una disminución de la concentración del pico máximo en la curva de lixiviación y una disminución del porcentaje del compuesto lixiviado debido al aumento de su adsorción. Por otra parte, el penconazol se mostró inmóvil, siendo retenido en el primer tramo de la columna. El proceso de adsorción contribuye a prevenir la degradación al no observarse ningún metabolito en los lixiviados.

El estudio del mecanismo de disipación de pesticidas en un suelo enmendado con SMS, indicó también la influencia de los distintos residuos en la mineralización de los pesticidas y en la formación de residuos enlazados al suelo (Marín-Benito y col., 2014, 2015).

Herrero-Hernández y col. (2011) estudiaron la disipación de tebuconazol en campo en un suelo enmendado con SMS. La distribución del tebuconazol a través del perfil del suelo (0-50 cm) indicó la movilidad del pesticida hacia capas más profundas en los casos del suelo enmendado, revelando la influencia de la materia orgánica (sólida y disuelta) aportada por la enmienda.

Recientemente Herrero-Hernández y col. (2015) mostraron también la influencia del SMS sobre la disipación del azoxistrobin, siendo esta más rápida en suelos no enmendados. Los experimentos se llevaron a cabo tanto en campo como en laboratorio, observando una menor velocidad de disipación en las condiciones de laboratorio. La aplicación de la enmienda afectó tanto a la adsorción como a la movilidad del azoxistrobin. La disipación fue más rápida bajo condiciones ambientales y la enmienda aumentó la persistencia del pesticida en las dos condiciones estudiadas.

En otros estudios existentes en la bibliografía mundial se han estudiado la influencia del SMS en la degradación y/o mineralización de compuestos orgánicos tales como benceno (Semple y col., 1998) e hidrocarburos aromáticos policíclicos (PAHs) (Li y col., 2010; García-Delgado y col., 2015b). Otros trabajos han estudiado su capacidad de eliminar contaminantes orgánicos como pentaclorofenol en sistemas acuosos (Law y col., 2003).

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Directiva 76/464/CEE, Directiva del Consejo, de 12 de junio de 1986, relativa a los valores límite y los objetivos de calidad para los residuos de determinadas sustancias peligrosas comprendidas en la lista I del Anexo de la Directiva 76/464/CEE.

Directiva 2000/60/CE del Parlamento Europeo y del Consejo, de 23 de octubre de 2000, por la que se establece un marco comunitario de actuación en el ámbito de la política de aguas.

Directiva 2006/118/CE del Parlamento Europeo y del Consejo, de 12 de diciembre de 2006, relativa a la protección de las aguas subterráneas contra la contaminación y el deterioro.

Directiva 2008/105/CE del Parlamento Europeo y del Consejo, de 16 de diciembre de 2008, relativa a las normas de calidad ambiental en el ámbito de la política de aguas, por la que se modifican y derogan ulteriormente las Directivas 82/176/CEE, 83/513/CEE, 84/156/CEE, 84/491/CEE y 86/280/CEE del Consejo, y por la que se modifica la Directiva 2000/60/CE.

Real Decreto 9/2005, de 14 de enero, por el que se establece la relación de actividades potencialmente contaminantes del suelo y los criterios y estándares para la declaración de suelos contaminados

Real Decreto Legislativo 1/2001, de 20 de julio, por el que se aprueba el texto refundido de la Ley de Aguas.

2. OBJETIVOS

2. OBJETIVOS

El objetivo principal del trabajo fue conocer la posible contaminación por pesticidas de suelos y aguas situados en zonas de viñedos de La Rioja mediante la evaluación de residuos de estos compuestos y desarrollar estrategias o métodos fisicoquímicos basados en la modificación del suelo con residuo postcultivo de champiñón (SMS), para la prevención y/o control de esta contaminación a partir del conocimiento de la dinámica de estos compuestos en los suelos.

Para conseguir este objetivo se abordaron los siguientes objetivos parciales:

1. Evaluación de la contaminación de suelos y aguas por pesticidas en zonas de viñedo de La Rioja: 1.1. Elaboración de un protocolo de extracción de los residuos de pesticidas de aguas y suelos, 1.2. Elaboración de un protocolo de análisis multiresiduo para la separación y análisis de compuestos con diferentes características, 1.3. Aplicación de protocolos al análisis de muestras de aguas y suelos reales.
2. Uso de sustrato postcultivo de champiñón como estrategia fisicoquímica de prevención y/o control de la contaminación: 2.1. Estudio de la adsorción/inmovilización de pesticidas con diferentes características, seleccionados por su frecuente presencia en las aguas, por suelos modificados con SMS a distintas dosis, 2.2. Evaluación de la disipación y biodisponibilidad de fungicidas inmovilizados en suelos modificados con dos dosis de SMS, 2.3. Determinación de la movilidad (lavado/retención) de fungicidas en columnas de suelo modificado con dos dosis de SMS.
3. Efecto de la aplicación simultánea de pesticidas y sustrato postcultivo de champiñón en las comunidades microbianas del suelo: 3.1. Evaluación mediante técnicas de biología molecular de los cambios en las comunidades eucariotas de un suelo agrícola por la aplicación simultánea de pesticidas y SMS.

Los Resultados obtenidos en el desarrollo de estos Objetivos Parciales han dado lugar a una serie de artículos originales de investigación, publicados, en proceso de revisión o en preparación, en revistas científicas de prestigio internacional e indexadas en la edición científica del *Journal Citation Reports* que se incluyen en el Apartado **5. ANEXOS** de esta Memoria en la versión publicada.

Un resumen en español de estos artículos describiendo los apartados de Materiales, Métodos Experimentales y Resultados y Discusión se incluyen en el Apartado **3. RESULTADOS Y DISCUSIÓN** de esta Memoria.

3.RESULTADOS Y DISCUSIÓN

3. RESULTADOS Y DISCUSIÓN

3.1. EVALUACIÓN DE LA CONTAMINACIÓN DE SUELOS Y AGUAS POR PESTICIDAS EN ZONAS DE VIÑEDO DE LA RIOJA

3.1.1. Descripción del área de estudio

El área de estudio está localizada en la Comunidad Autónoma de la Rioja, en zonas de viñedo pertenecientes a la Denominación de Origen Calificada Rioja (DOCa), situada en el Norte de España, a ambos márgenes del río Ebro. Los accidentes geográficos han contribuido a conformar una región natural perfectamente delimitada y diferenciada de las que le rodean, si bien administrativamente las 63593 hectáreas de viñedo protegidas actualmente por la Denominación se distribuyen en el territorio de las tres provincias ribereñas (Figura 7) del curso alto del Ebro: La Rioja (43885 ha), Álava (12934 ha) y Navarra (6774 ha) (García-Santa María, 2004).

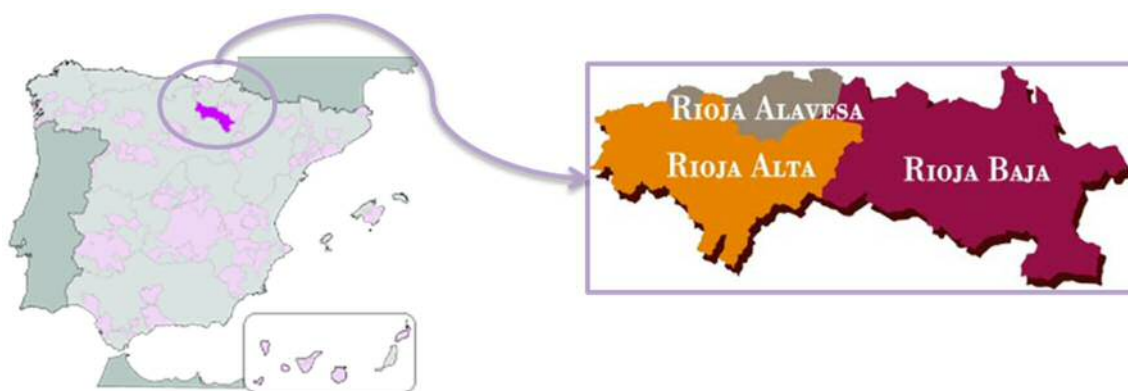


Figura 7. Localización geográfica del área de estudio.

Cien kilómetros de distancia separan Haro, la localidad más occidental, de Alfaro, la más oriental, siendo de unos 40 kilómetros la anchura máxima del valle ocupado por viñedos que, en sucesivas terrazas escalonadas, alcanzan una altitud máxima de unos 700 m. Con escasas modificaciones en los últimos treinta años, el Reglamento de la Denominación enumera los 144 términos municipales concretos (118 en La Rioja, 18 en Álava y 8 en Navarra) en los que se ubican "los terrenos que el Consejo Regulador considera aptos para la producción de uva con la calidad necesaria".

Toda la zona se beneficia de la confluencia de dos climas tan opuestos como el atlántico y mediterráneo, que proporcionan temperaturas suaves y precipitaciones anuales algo superiores a los 400 L/m², condiciones muy idóneas para el desarrollo de la vid. El tipo de suelo más característico de la Rioja también resulta muy adecuado para una viticultura de calidad, ya que tiene una estructura equilibrada (arenas, limos y arcillas), es ligeramente alcalino, pobre en materia orgánica y con moderada disponibilidad hídrica durante el verano. El propio reglamento reconoce la existencia de tres subzonas con características vitivinícolas diferenciadas: en Rioja Alavesa predominan la influencia del clima atlántico y los suelos arcillo calcáreos situados en terrazas y pequeñas parcelas; en Rioja Alta también predomina el clima atlántico, mientras que sus suelos se reparten entre los arcillo-calcáreos, arcillo-ferrosos y aluviales; y la Rioja Baja posee un clima más seco y cálido debido a la influencia mediterránea, predominando en ella los suelos aluviales y arcillo-ferrosos.

La agricultura de La Rioja tiene una tendencia hacia el monocultivo sustituyendo al policultivo tradicional. Sólo dos cultivos han visto aumentada su masa forestal desde el año 1990: la vid que ha crecido el 26% y el olivar con un incremento del 331%. El cereal sigue siendo el cultivo con más superficie, seguido del viñedo, los frutos secos, las hortalizas y el olivar (www.larioja.org). De acuerdo con los rendimientos máximos permitidos por el Reglamento a fin de optimizar la calidad de los vinos (6500 kg/ha en variedades tintas y 9000 kg/ha en variedades blancas), la producción media actual de la Denominación se sitúa entre 280 y 300 millones de litros anuales, de los que el 90% corresponde a vino tinto y el resto a blanco y rosado.

3.1.2. Materiales

- **Selección de pesticidas**

Se seleccionaron para el estudio una serie de pesticidas con actividad insecticida, fungicida y herbicida. La selección se llevó a cabo entre aquellos compuestos más utilizados en los cultivos de viñedo de la Rioja. Inicialmente, para el estudio se obtuvo información a partir de datos estadísticos publicados por la AEPLA, empresas que comercializan los pesticidas y la Consejería de Agricultura del Gobierno de la Rioja. Se consideró también la información facilitada por viticultores de la zona y especialmente

por el personal de las bodegas Dinastía Vivanco y CVNE que aportaron el calendario de actuación y los productos aplicados.

En la Tabla 4 se incluyen los compuestos seleccionados para el estudio, junto con los productos de degradación estudiados. Los compuestos puros fueron suministrados por Riedel de Haën (Seelze-Hannover, Germany) y Dr. Ehrenstorfer (Augsburg, Germany) (pureza>98%) y se utilizaron sin ninguna purificación adicional.

Tabla 4. Pesticidas y productos de degradación analizados en aguas (mediante GC-MS (1) o LC-MS (2)) y en suelos (3) de La Rioja. Los compuestos con letras entre paréntesis corresponden a los productos de degradación de los pesticidas con las mismas letras como superíndices.

| Pesticida | Uso | Solubilidad en agua (mg L ⁻¹) | Índice GUS | Log Kow | K _{oc} | DT50 (días) |
|------------------------------|-------------|---|------------|---------|-----------------|-------------|
| ¹ Simazina | Herbicida | 5 | 2.00 | 2.3 | 130 | 60 |
| ¹ Alacloro | Herbicida | 240 | 1.08 | 3.09 | 335 | 14 |
| ¹ Terbutrina | Herbicida | 25 | 2.40 | 3.66 | 2432 | 74 |
| ² Clorsulfuron | Herbicida | 12500 | 5.38 | -0.99 | - | 160 |
| ^{2,3} CGA-62826 (a) | Metabolito | - | - | - | - | - |
| ² Flazasulfuron | Herbicida | 2100 | 2.34 | -0.06 | 46 | 10 |
| ² DIHA (b) | Metabolito | - | - | - | - | - |
| ² Acefato | Insecticida | 790000 | 1.14 | -0.85 | 302 | 3 |
| ² DEHA (c) | Metabolito | - | - | - | - | - |
| ² Cimoxanilo | Fungicida | 780 | -0.37 | 0.67 | - | 0.7 |
| ² Pirimidinol (d) | Metabolito | - | - | - | - | - |
| ^{1,2} DIA (e) | Metabolito | 670 | - | - | - | - |
| ² Imidacloprid | Insecticida | 610 | 3.76 | 0.57 | - | 191 |
| ² Cloridazon | Herbicida | 422 | 2.54 | 1.19 | 120 | 31 |
| ^{1,2} Dimetoato | Insecticida | 39800 | 1.06 | 0.70 | - | 2.6 |
| ^{1,2} Metamitrona | Herbicida | 1770 | 3.09 | 0.85 | 77.7 | 30 |
| ² HA (f) | Metabolito | - | - | - | - | - |
| ^{1,2} DEA (g) | Metabolito | 3200 | 3.54 | - | - | - |
| ^{1,2} CGA 92370 (h) | Metabolito | - | - | - | - | - |
| ² Carbendazima | Fungicida | 8.0 | 2.64 | 1.48 | - | 40 |
| ² Carbofurano | Insecticida | 322 | 3.02 | 1.8 | - | 29 |
| ^{2,3} HT (i) | Metabolito | 7.19 | 4.59 | - | - | - |
| ^{1,2} Metribuzina | Herbicida | 1165 | 2.57 | 1.65 | - | 11.5 |
| ² CMPU (j) | Metabolito | - | - | - | - | - |
| ^{1,2,3} DET (k) | Metabolito | 327.1 | 3.54 | - | - | - |

3. Resultados y discusión

| | | | | | | |
|---|-------------|-------|-------|------|--------|------|
| ² Carbaril | Insecticida | 9.1 | 2.02 | 2.36 | 300 | 16 |
| ^{1,2,3} Flutriafol | Fungicida | 95.0 | 5.40 | 2.3 | - | 1358 |
| ^{1,2,3} Lenacilo | Herbicida | 2.9 | 2.18 | 1.69 | 165 | 179 |
| ² Isoproturon | Herbicida | 70.2 | 2.07 | 2.5 | - | 12 |
| ² Clorotoluron ^j | Herbicida | 74 | 2.79 | 2.5 | - | - |
| ^{1,2,3} Metalaxil ^{a,h} | Fungicida | 8400 | 2.11 | 1.65 | 162 | 36 |
| ^{1,2} Atrazina ^{b,c,e,f,g} | Herbicida | 35 | 3.30 | 2.7 | 100 | 75 |
| ^{1,2} Metobromuron | Herbicida | 330 | 2.52 | 2.41 | - | - |
| ^{1,2,3} Fluometuron | Herbicida | 111 | 3.92 | 2.28 | - | 63.5 |
| ^{1,2,3} Pirimicarb | Insecticida | 3100 | 2.73 | 1.70 | - | 86 |
| ² Diuron | Herbicida | 35.6 | 1.83 | 2.87 | 813 | 75.5 |
| ^{1,2} Nuarimol | Fungicida | 26 | 3.52 | 3.18 | 241 | 150 |
| ² Ciproconazol | Fungicida | 93 | 3.25 | 3.09 | - | 142 |
| ² Azoxiestrobina | Fungicida | 6.0 | 2.53 | 2.5 | 589 | 78 |
| ^{1,2} Propazina | Herbicida | 8.6 | 3.84 | 3.95 | 154 | 131 |
| ^{1,2,3} Miclobutanilo | Fungicida | 132 | 3.54 | 2.89 | - | 560 |
| ^{1,2,3} Terbutilazina ^{b,e,i,k} | Herbicida | 6.6 | 3.07 | 3.4 | - | 75.1 |
| ^{1,2,3} Etofumesato | Herbicida | 50 | 3.38 | 2.7 | - | 70 |
| ² Iprovalicarb | Fungicida | 17.8 | 2.35 | 3.2 | 106 | 15.5 |
| ^{1,2,3} Triadimenol | Fungicida | 72.0 | 3.75 | 3.18 | 750 | 250 |
| ^{1,2,3} Pirimetanil | Fungicida | 121 | 2.65 | 2.84 | - | 55 |
| ^{2,3} Metoxifenoazida | Insecticida | 3.3 | 3.02 | 3.72 | 402 | 146 |
| ² Linuron | Herbicida | 63.8 | 2.03 | 3.0 | 739 | 48 |
| ² Dimetomorf | Fungicida | 28.95 | 2.56 | 2.68 | - | 57 |
| ^{1,2} Acetoclor | Herbicida | 282 | 1.77 | 4.14 | 156 | 14 |
| ^{1,2,3} Metolaclo | Herbicida | 530 | 3.49 | 3.4 | 120 | 90 |
| ^{1,2} Penconazol | Fungicida | 73.0 | 1.51 | 3.72 | - | 117 |
| ^{1,2,3} Kresoxim-metil | Fungicida | 2.0 | 1.82 | 3.4 | - | 16 |
| ^{1,2,3} Tebuconazol | Fungicida | 36.0 | 2.0 | 3.7 | - | 63 |
| ^{1,2} Benalaxil | Fungicida | 28.6 | 0.51 | 3.54 | 4998 | 49 |
| ^{1,2} Ciprodinil | Fungicida | 13 | 1.2 | 4.0 | - | 37 |
| ^{1,2} Diazinon ^d | Insecticida | 60 | 1.14 | 3.69 | 609 | 9.1 |
| ² Trifloxiestrobina | Fungicida | 0.61 | 0.53 | 4.5 | - | 7 |
| ^{1,2} Diclofop-metil | Herbicida | 0.39 | 0 | 4.8 | - | 1 |
| ^{1,2} Clorpirifos | Insecticida | 1.05 | 0.15 | 4.7 | 8151 | 50 |
| ² Cipermetrin | Insecticida | 0.009 | -2.12 | 5.3 | 156250 | 60 |

PPDB, Pesticide Properties DataBase (2015). Solubilidad en agua a 20 °C, log Kow coeficiente de partición octanol/agua a pH 7 y 20°C, Koc coeficiente de adsorción normalizado al contenido de carbono orgánico, DT50 Vida media del compuesto en suelo.

- **Toma y preparación de muestras de aguas**

Las muestras de agua se localizaron en las tres diferentes sub-áreas de La Rioja (Rioja Alavesa, Rioja Alta y Rioja Baja) que agrupa la Denominación de Origen Calificada Rioja. En un primer muestreo realizado en marzo de 2010 se tomaron 25 muestras y se analizaron 34 compuestos, seleccionados entre los pesticidas agrícolas más comúnmente utilizados, mediante cromatografía de gases con detección por espectrometría de masas. (GC-MS). El segundo muestreo tuvo lugar en marzo de 2011, se recogieron un total de 92 muestras y se analizaron 58 compuestos entre insecticidas, herbicidas y fungicidas y algunos de sus productos de degradación mediante cromatografía de líquidos con detección por espectrometría de masas (LC-MS). Las muestras correspondientes a aguas subterráneas se tomaron en pozos privados, en fuentes públicas o en manantiales. En la Figura 8 se muestran algunos puntos de muestreo. Las profundidades de estos pozos varían entre 1 y 15 m, únicamente tres de ellos tenían profundidades entre 17 y 60 m. Los pozos muestreados se encuentran en zonas de cultivo y de ellos se toma habitualmente el agua para el riego. Dependiendo de la profundidad de estos pozos, las muestras se tomaron manualmente o mediante bomba.

La localización de las muestras tomadas en marzo 2011 así como los detalles de su situación y proximidad a cultivos determinados se muestran en Figura 1 y Tabla 2 incluidas en Anexos de esta Memoria (Páginas 141-143).

Se tomaron muestras de aguas superficiales y subterráneas. Las muestras fueron recogidas por duplicado en botellas de cristal de color ámbar para evitar las alteraciones con la luz y se transportaron refrigeradas al laboratorio. Todas las muestras fueron filtradas inmediatamente utilizando filtros de nitrocelulosa (Millipore) y permanecieron almacenadas en la cámara fría a 4 °C hasta su análisis que tuvo lugar en un periodo inferior a una semana.



Figura 8. Ejemplos de puntos de muestreo de aguas

- **Toma y preparación de muestras de suelos**

El muestreo de suelos se realizó en marzo del 2012, recogándose un total de 17 muestras de suelo pertenecientes a las distintas sub-áreas de la Rioja. Los puntos de muestreo fueron seleccionados en función de los resultados del análisis de pesticidas en aguas mediante LC-MS, realizado en marzo del 2011. En la medida de lo posible los suelos se localizaron alrededor de las captaciones de agua, a una distancia entre 50 y 100 m del punto de muestreo del agua. Las muestras se transportaron en bolsas de plástico y se mantuvieron en la oscuridad a 4°C hasta su análisis. Cada muestra fue analizada por triplicado después de optimizar el protocolo de extracción.

Tanto para la optimización de la extracción de pesticidas en suelo como para la validación del método analítico se utilizaron suelos que no contenían ninguno de los compuestos estudiados. Estos blancos de suelo se tomaron del horizonte superficial (0-20 cm) en la finca experimental Muñovela del IRNASA-CSIC (Salamanca) y en un viñedo en la localidad de Valdenebro (Valladolid). Las muestras se secaron y tamizaron (< 2 mm) y sus características fisicoquímicas se determinaron siguiendo los métodos analíticos estándar (MAPA, 1986) (Tabla 2 en Página 162 de Anexos).

3.1.3. Métodos Experimentales

Se elaboró un protocolo de análisis para la separación y determinación de todos los compuestos seleccionados en muestras de agua y suelos, aplicándose posteriormente al análisis de los compuestos en muestras reales de agua y suelos. Para llevar a cabo este protocolo se prepararon disoluciones que contenían todos los pesticidas en concentraciones de 1000 o 500 $\mu\text{g/mL}$, disolviendo estándares de referencia en metanol y a partir de estas disoluciones se prepararon otras disoluciones conteniendo todos los compuestos diluyendo alícuotas en metanol. Estas disoluciones fueron almacenadas a 4°C en la oscuridad y fueron utilizadas para la preparación de disoluciones estándar de trabajo de concentraciones adecuadas que pudieran ser utilizadas en el análisis de las muestras de aguas y de suelos.

Se optimizó el procedimiento de concentración de aguas mediante el método de extracción en fase sólida utilizando distintos cartuchos y de extracción de suelos utilizando distintos disolventes y el procedimiento de análisis utilizando las técnicas de GC-MS y/o LC-MS.

- **Optimización del procedimiento de extracción de pesticidas de las aguas**

Para detectar las concentraciones establecidas por la legislación de la Unión Europea (98/83/EC, 2006/118/EC) en aguas de bebida y subterráneas ($< 0.1 \mu\text{g L}^{-1}$) menores que el límite de detección de la técnica utilizada, fue necesario llevar a cabo la pre-concentración de las muestras. La optimización de esta etapa es crítica ya que se puede producir una pérdida del analito o inducir a una contaminación externa de la muestra. Para llevar a cabo la pre-concentración de los pesticidas se utilizó la técnica de extracción en fase sólida (SPE) optimizando el tipo de sorbente de la fase sólida, el disolvente empleado y el volumen de muestra a concentrar.

Se ensayaron los siguientes cartuchos para este proceso: cartuchos poliméricos Oasis HLB (60 mg, Waters), LiChrolut En (200 mg, Merk) y Strata-X (60 mg, Phenomenex), cartuchos de C_{18} soportado en Si (Sep-Pak Plus 900 mg, Waters) y cartuchos de carbón (SamplinQ 500 mg, Agilent). Cada cartucho fue acondicionado previamente. Para calcular las recuperaciones se pasaron a través de cada tipo de cartucho 50-100 mL de agua ultrapura con una concentración de $1-4 \mu\text{g L}^{-1}$ de cada

compuesto a una velocidad de 7 mL min^{-1} . Finalmente cada cartucho con el analito retenido se sometió a una corriente de vacío (-20 mm Hg) durante 5 minutos para su secado. Los analitos retenidos en el cartucho se eluyeron con metanol. La fase orgánica obtenida se evaporó a sequedad mediante una corriente de nitrógeno a 45°C en un evaporador EVA-EC2-L (VLM GmbH, Bielefeld, Alemania). El residuo obtenido se disolvió en 0.5 mL de metanol (análisis GC-MS) o metanol:agua (50:50) (análisis LC-MS) y se procedió a su análisis después de filtrarlo a través de filtros con un tamaño de poro de $0.45 \mu\text{m}$ (GHP Acrodisc, Waters Corporation).

Una vez seleccionado el tipo de cartucho se estudió la influencia del eluyente. Para ello se analizaron cuatro disolventes: metanol, acetonitrilo, acetona y hexano. Los extractos se obtuvieron como se ha descrito anteriormente, aunque acondicionando los cartuchos con el disolvente correspondiente y agua ultrapura.

El último parámetro a optimizar fue el volumen de muestra eluido a través de los cartuchos. Para ello se ensayaron diferentes volúmenes de agua ultrapura (50, 100, 200, 500 y 1000 mL) con la misma cantidad de pesticida ($0.2 \mu\text{g}$). Los extractos se obtuvieron como se ha explicado anteriormente.

- **Optimización del método de extracción de pesticidas en suelos**

El primer paso a la hora de optimizar el protocolo de extracción multiresiduo de pesticidas en suelos fue la selección del disolvente. Se probaron disolventes individualmente (metanol, acetona, acetonitrilo, acetato de etilo y agua) para la extracción y también se probaron diferentes mezclas como metanol/acetona (50:50); metanol/ CaCl_2 0.01M (50:50) y metanol/agua (50:50) a pH 4, 7 y 9.

Los dos blancos de suelo seleccionados se contaminaron con una disolución de metanol conteniendo todos los pesticidas a estudiar utilizando dos dosis, una correspondiente a la dosis media de aplicación de estos compuestos (0.1 mg kg^{-1}) y otra a una dosis más alta (1 mg kg^{-1}). La mezcla del suelo con la disolución permaneció a temperatura ambiente durante 24 h con el fin de evaporar el metanol. Para estudiar la influencia del posible envejecimiento de los compuestos en el suelo se realizó el mismo proceso indicado anteriormente y la extracción se llevó a cabo después de 7, 15 y 30 días de permanencia en el suelo de los compuestos.

Para la extracción de los pesticidas se agitaron por triplicado 5 g de suelo con 10 mL del disolvente durante 24 h en una cámara termostatzada a 20 °C, posteriormente la suspensión se centrifugó a 3100 x g durante 15 min y 6 mL de extracto se llevaron a sequedad bajo una corriente de nitrógeno a 45°C en un evaporador EVA_EC2-L (VLM, Bielefeld, Alemania). Cuando se utilizaron como extractantes mezclas metanol/agua o metanol/CaCl₂ 0.01 M, fue necesario realizar una SPE para eliminar la fase acuosa. En este caso, el cartucho utilizado fue Oasis HLB (siguiendo el protocolo optimizado para el análisis de aguas descrito anteriormente). Se llevó a cabo previamente el acondicionamiento del cartucho y después de la retención de los analitos, los cartuchos se llevaron a sequedad y se eluyeron con 4 mL de acetonitrilo y posteriormente con 4 mL de acetona. Tras la evaporación de la fase orgánica, el residuo se disolvió en 0.5 mL de metanol, se filtró a través de un filtro de 0.45 µm (GHP acrodisc, Waters Corporation) y se analizó mediante GC-MS. Para el análisis mediante LC-MS, 0.1 mL de la muestra utilizada en el análisis de GC-MS se diluyó con agua hasta un volumen de 0.2 mL para obtener el medio de inyección adecuado, una mezcla metanol/agua (1:1).

- **Determinación de pesticidas en aguas y extractos de suelos mediante cromatografía de gases con detector de espectrometría de masas (GC-MS)**

El análisis de los pesticidas por GC-MS se realizó en un Cromatógrafo de Gases Agilent 7890 acoplado a un espectrómetro de masas Agilent 5975 MSD (Agilent Technologies, Wilmington, DE, USA). Se empleó una columna cromatográfica de 30 m, con un diámetro interno de 0.25 mm y un espesor de partícula de 0.25 µm de DB-17MS (J&W, Avondale, USA).

Las condiciones óptimas de trabajo del equipo para el análisis de pesticidas en aguas fueron las siguientes: el espectrómetro de masas estaba equipado con una fuente de ionización por impacto de iones, con una energía de ionización de 70 eV y se fijó en 1700 V el voltaje del multiplicador y un tiempo de residencia de 10 mseg, la temperatura de la fuente y de la línea de transferencia fueron 230°C y 280°C respectivamente; el gas portador fue helio a una velocidad de flujo de 1.5 mL min⁻¹. La inyección se llevó a cabo en modo splitless inyectando 1 µL a 220°C. En el modo splitless la mayoría de muestra inyectada entra a la columna, siendo ideal para la detección de trazas. La rampa de temperatura del horno se programó para obtener todos

los compuestos dentro de una ventana de 24 min, incrementándose desde 100 a 200°C con una velocidad de 20°C min⁻¹ después de un tiempo de permanencia de 1 min, posteriormente se incrementó a 210°C a 1°C min⁻¹, a 230°C a 5°C min⁻¹ y a 315°C a 50°C min⁻¹ (2-3 min).

La detección de los iones se realizó en modo SIM (Selected Ion Monitoring) de acuerdo con los parámetros indicados en la Tabla 1 de Anexos (Página 124) de esta Memoria. De cada compuesto se seleccionaron las tres masas más abundantes, de las cuales una de ellas fue elegida para la cuantificación, verificando siempre el tiempo de retención y la intensidad del pico cromatográfico.

Las condiciones del equipo de GC-MS para la determinación de los pesticidas en extractos de suelos fueron las mismas que las establecidas para la determinación de pesticidas en muestras de agua con la diferencia del modo de operación del inyector, que en este caso fue con purga de disolvente (solvent vent mode) a 60°C. La temperatura del horno se programó siguiendo el siguiente esquema: desde la temperatura inicial (60°C) se incrementó a 100°C con una velocidad de 40°C min⁻¹, posteriormente se incrementó a 220°C (20°C min⁻¹), seguido de una tercera rampa a 1°C min⁻¹ hasta 250°C. Finalmente con un incremento de 10°C min⁻¹ se llegó hasta 310°C, permaneciendo a esta temperatura 2 min. El tiempo de análisis por muestra fue 32 min. Al igual que en el análisis de aguas, se seleccionaron las tres masas más abundantes de cada compuesto y una de ellas fue la que se utilizó para la cuantificación.

La cuantificación se llevó a cabo mediante calibración externa. Las curvas de calibración fueron obtenidas representando el área de cada pico frente a la concentración. Los patrones para la obtención de las curvas de calibración se prepararon en extractos de suelo no contaminados siguiendo el mismo protocolo de extracción que para las muestras de suelo con pesticida.

- **Determinación de pesticidas en aguas y extractos de suelos mediante cromatografía líquida y detector de espectrometría de masas (LC-MS)**

El análisis de los pesticidas por LC-MS se llevó a cabo en un equipo de Cromatografía Líquida de Alta Resolución (HPLC Waters, Milford, MA, USA), equipado con un sistema de bombeo multisolvente modelo e2695 con inyector

automático, acoplado en serie con un espectrómetro de masas Micromass-ZQ (MS) equipado con una interfaz de electrospray (ESI) y un sistema de adquisición y procesamiento de datos Empower Pro de Waters Technologies.

Las condiciones del detector fueron las siguientes: el voltaje del capilar se fijó en 3.1 kV; la temperatura de la fuente, del cono y la de solvatación utilizadas fueron 120, 20 y 300 °C, respectivamente; la velocidad del gas de solvatación fue 400 L h⁻¹ y la del gas del cono 60 L h⁻¹. La columna utilizada fue una Luna PFP2 (Phenomenex, Torrance, CA, USA) de 150 mm x 4.60 mm d.i. y 3.0 µm de tamaño de partícula, acoplada a una pre-columna de C-18 de Waters (Milford, MA, USA). La fase móvil fue metanol y agua acidificada con formiato amónico (5 mM) a pH 5.

El gradiente de elución fue el siguiente: la composición de la fase móvil inicial fue 60% de metanol, incrementándose linealmente en 4 min hasta el 75%, manteniéndose constante durante 3 min. Se alcanzó el 80% de metanol en 4 min manteniéndose constante durante 3 min. El porcentaje de metanol se incrementó hasta el 100% en 6 min y se mantuvo constante otros 5 min, para volver a las condiciones iniciales en 2 min. La columna se equilibró durante 5 min y la velocidad de flujo fue 0.3 mL min⁻¹. El volumen inyectado fue 20 µL.

Para optimizar la detección de todos los pesticidas se inyectaron los patrones de cada pesticida directamente en el equipo operando en modo scan (rango m/z 50-500). La cuantificación se llevó a cabo mediante un calibrado externo. Estas curvas de calibración fueron obtenidas a partir del área bajo el pico de cada compuesto obtenido en modo SIM. Estas rectas de calibrado se realizaron con muestras de agua no contaminadas, a las que se les añadieron los analitos para evitar el posible efecto matriz. Con el fin de comprobar el efecto memoria se inyectó un blanco cada 55 muestras.

Las condiciones utilizadas en el equipo de LC-MS para la determinación de pesticidas en los extractos de suelos fueron las mismas que para el estudio de las aguas con la excepción del gradiente, que fue el siguiente: la fase móvil comenzó con un 60% de metanol, incrementándose al 80% en 3 min y manteniéndose constante durante 6 min. El porcentaje de metanol aumentó hasta el 100% en 15 min manteniéndose constante 4 min, para finalmente volver a las condiciones iniciales durante 4 min. El tiempo total de análisis para la detección de todos los compuestos fue 32 min. El flujo

establecido fue 0.3 mL min^{-1} y el volumen de inyección $20 \text{ }\mu\text{L}$. La optimización se realizó en modo scan (rango m/z 50-500).

- **Validación del método de determinación de pesticidas en aguas**

Con el fin de evaluar la influencia del efecto matriz en el análisis se realizó el protocolo de pre-concentración anteriormente explicado con tres tipos diferentes de agua: ultrapura (UHQ), superficial (río) y subterránea utilizando volúmenes de 500 mL dopados con todos los compuestos a la misma concentración ($0.2 \text{ }\mu\text{g L}^{-1}$). Las muestras de agua fueron previamente analizadas para verificar la ausencia de los analitos estudiados. Después de la pre-concentración, la extracción y el análisis se compararon los resultados de la muestra de agua de río y de agua superficial con el resultado de la muestra de agua ultrapura.

Además, la validación del método se realizó para cada compuesto de forma individual, estudiando la exactitud del método y la precisión al nivel de concentración establecido por la legislación de la Comunidad Europea y los límites de detección y cuantificación. Para estimar la linealidad y los límites de detección (LOD) y cuantificación (LOQ), se prepararon ocho patrones de 500 mL de agua subterránea (sin presencia de analitos estudiados), conteniendo todos los analitos en un rango de concentración de 0.1 a $2 \text{ }\mu\text{g L}^{-1}$ en el LC-MS y de 0.1 a $1.5 \text{ }\mu\text{g L}^{-1}$ para el GC-MS. Para evaluar tanto la exactitud como la precisión del método se prepararon muestras de agua subterránea libre de pesticidas conteniendo todos los compuestos a analizar en dos concentraciones diferentes (0.1 y $0.5 \text{ }\mu\text{g L}^{-1}$). Las recuperaciones se obtuvieron analizando seis réplicas. Los límites de detección (LODs) y cuantificación (LOQs) para cada analito fueron estimados como la concentración del analito con una relación señal/ruido de 3 y 10 respectivamente.

- **Validación del método de determinación de pesticidas en suelos**

Para evaluar tanto la exactitud como la precisión de este método se analizaron las recuperaciones de las extracciones de 5 muestras de suelo no contaminado al que se le añadió 0.1 mg kg^{-1} de cada pesticida. La linealidad de las curvas de calibración fueron evaluadas para todos los pesticidas midiendo 8 concentraciones preparadas en extracto proveniente de suelo no contaminado. El rango de concentraciones medidas

fueron $0.005\text{-}1\ \mu\text{g mL}^{-1}$ (correspondientes a $0.835\text{-}167.1\ \mu\text{g kg}^{-1}$ para GC-MS y a $1.667\text{-}333.4\ \mu\text{g kg}^{-1}$ para LC-MS). Los límites de detección (LODs) y cuantificación (LOQs) fueron estimados como la concentración del analito con una relación señal/ruido de 3 y 10 respectivamente.

3.1.4. Evaluación de residuos de pesticidas en aguas de zonas de viñedo de La Rioja

Como fase inicial para llevar a cabo el estudio de la evaluación de residuos de pesticidas en aguas de zonas de viñedo de la Rioja fue necesario el desarrollo de un método de análisis multiresiduo para poder detectar un número elevado de compuestos. En una primera aproximación se puso a punto un método para la determinación de un número limitado de pesticidas en una serie pequeña de muestras de aguas procedentes de zonas con alto riesgo de contaminación por pesticidas, basado en una extracción en fase sólida (SPE) seguida por un procedimiento analítico mediante GC-MS. Posteriormente la evaluación de residuos de pesticidas se extendió a un número más amplio de muestras debido a las posibilidades de muestreo en el área de interés y a un número más elevado de pesticidas de acuerdo con la información aportada por agricultores de la zona. Los análisis de los pesticidas en esta segunda fase de muestreo se llevaron a cabo siguiendo el método de pre-concentración puesto a punto pero se utilizó el método analítico de LC-MS por su capacidad para analizar un mayor número de compuestos.

3.1.4.1. Evaluación de residuos de pesticidas en aguas mediante GC-MS

Se desarrolló un método de análisis multiresiduo para poder detectar 34 pesticidas en aguas basado en una extracción en fase sólida seguida de un análisis mediante un equipo de cromatografía de gases acoplado a un detector de espectrometría de masas (GC-MS).

El primer paso fue la optimización de las condiciones cromatográficas, donde se evaluaron todos los parámetros que podían afectar tanto a la resolución como al tiempo de análisis. Se optimizó el programa de temperaturas del horno y el flujo del gas portador. Así mismo se ensayaron diferentes modos de inyección, observando que no había diferencias entre operar en modo split o splitless y pulsed-splitless. Finalmente el modo splitless fue seleccionado para la inyección de la muestra. Una vez optimizados todos los parámetros del equipo cromatográfico se pasó a seleccionar los iones con los que se cuantificaron e identificaron los compuestos analizados, para ello se comparó el espectro obtenido de cada compuesto con el espectro procedente de la librería (NIST 08). Esta selección se realizó en función de la abundancia que presentaban y/o si eran característicos de cada analito en cuestión. En la Tabla 1 de Anexos, página 124, se incluyen los iones seleccionados para cada compuesto.

Para la elaboración del protocolo de SPE se estudiaron distintos tipos de sorbentes con distinta naturaleza química utilizados en la etapa de preconcentración. De los cuatro cartuchos estudiados (Oasis HLB, LiChrolut En, C-18 y cartuchos de grafito) se seleccionaron Oasis HLB y LiChrolut EN, debido a que los porcentajes de recuperación fueron muy similares, entre 42% y 97%. Las recuperaciones más altas para algunos compuestos como triazinas, metoalacoloro y nuarimol se obtuvieron con Oasis HLB (Figura 1 de Anexos página 127).

Una vez seleccionados los cartuchos se procedió a seleccionar el mejor eluyente entre cuatro disolventes diferentes: metanol, acetonitrilo, acetona y hexano. Para ello se prepararon muestras de 50 mL con una concentración de $4 \mu\text{g L}^{-1}$ de cada compuesto que se hicieron pasar a través de los cartuchos. Se observó que las recuperaciones con hexano eran menores del 50%. Para el resto de disolventes se observaron diferentes comportamientos en función de la polaridad del compuesto. Las mejores recuperaciones para los analitos más polares se obtuvieron con acetonitrilo y metanol para Oasis y

LiChrolut respectivamente. Mientras que para los compuestos hidrofóbicos las mejores recuperaciones se obtuvieron con acetona para ambos cartuchos. Teniendo en cuenta estos resultados se consideró que la elución con dos disolventes (4 mL de acetona + 4 mL de acetonitrilo o metanol) fue la más adecuada para optimizar las recuperaciones de los residuos de los distintos pesticidas siendo mayores del 65% para todos los compuestos.

Para optimizar el factor de enriquecimiento y comprobar la ausencia del fenómeno de ruptura se contaminaron diferentes volúmenes (100, 250, 500 y 1000 mL) con la misma concentración de cada pesticida (0.2 µg) y se pasaron a través de cartuchos Oasis HLB. Los resultados indicaron que no se observaban diferencias en las recuperaciones independientemente del volumen de muestra estudiado. Únicamente cuando el volumen preconcentrado fue 1000 mL, se observó una disminución en las recuperaciones obtenidas para todos los analitos, siendo esta disminución mayor para diazinon y metamitron. Por esta razón, con el fin de obtener un equilibrio entre el incremento del factor de enriquecimiento y el tiempo del experimento y/o la aplicabilidad para un análisis de rutina, se fijó un volumen de muestra de 500 mL para la extracción de los residuos de pesticidas.

Finalmente, con el fin de evaluar el efecto matriz en el análisis y validar el método optimizado se analizaron simultáneamente, siguiendo el protocolo descrito una muestra de agua subterránea (exenta de pesticidas) y otra de agua ultrapura contaminadas con los analitos objeto de estudio. Al comparar las señales obtenidas para los pesticidas extraídos de ambas muestras, más del 85% de los compuestos registraron una supresión de la señal muy baja (del -15% al 15%). Los únicos compuestos que tuvieron variación en la señal en torno al 15-20% fueron CGA-92370, DET, miclobutanil, metribuzina y acetocloro. A pesar de que el efecto matriz observado fue pequeño, se usaron patrones dopados en la matriz para cuantificar de forma más precisa la concentración de estos compuestos en muestras reales de agua subterránea. Para ello los patrones se prepararon con 500 mL de una muestra de agua ambiental con concentraciones entre 0.1 y 1.5 µg/L. Se obtuvo una buena linealidad, con coeficientes de regresión (r^2) superiores a 0.99 para todos los compuestos en el rango estudiado. Únicamente el miclobutanil presentó un r^2 de 0.989.

El método fue validado calculando los límites de detección, límites de cuantificación, la exactitud y la precisión a niveles de concentración de pesticida entre 0.1 y 0.5 µg/L en muestras de agua ambiental no contaminada (Tabla 2 de Anexos, página 129). Los valores de recuperación fueron similares a los que se obtuvieron en la optimización de la extracción en fase sólida cuando la preconcentración se realizó con agua ultrapura, confirmando la ausencia del efecto matriz o su corrección cuando se usan patrones dopados en la matriz.

Una vez optimizado y validado el método para determinar pesticidas en muestras de agua mediante GC-MS, se procedió a aplicar el método desarrollado al análisis de 34 pesticidas y sus productos de degradación en 25 muestras de aguas subterráneas procedentes de zonas de viñedo de la Rioja (Tabla 3 de Anexos, página 131).

De los herbicidas analizados, tanto la terbutilazina como su metabolito DET fueron detectados en el 96% de las muestras analizadas siendo en algunos casos su concentración superior hasta 10 veces el límite establecido por la legislación Europea. Sin embargo, otros metabolitos de este mismo grupo como la desisopropyl atrazina-DIA solo se encontraron en menos del 20% de las muestras, o no fueron detectados en ninguna muestra como es el caso de DEA. Tanto DIA como DET son metabolitos de la terbutilazina pero el primero de ellos sólo se detectó en dos muestras y a concentraciones superiores a 0.1 µg/L. Esto es debido a que DET es el producto de degradación de la terbutilazina más abundante, mientras que DIA es un producto de degradación minoritario.

Otro resultado destacable es el hecho de que se encontraron en las muestras de agua subterránea pesticidas que fueron prohibidos por la Unión Europea en 2004, como la atrazina y la simazina, también de la familia de las triazinas. Metoalacoloro, etofumesato y fluometuron fueron otros herbicidas también detectados en más del 50% de las muestras.

Todos los fungicidas estudiados fueron detectados en al menos alguna de las muestras de agua. Los fungicidas que se detectaron en mayor número de muestras analizadas (> 50%) fueron: pirimetanil, ciprodinil, metalaxil, tebuconazol y kresoxim-metil. El metalaxil, su metabolito el CGA-92370 y el triadimenol fueron detectados en

concentraciones 10 veces superiores al límite establecido por la legislación ($0.1 \mu\text{g L}^{-1}$ para pesticidas individuales).

Los insecticidas incluidos en este estudio se detectaron en menores concentraciones y en menor número de muestras. Hay que tener en cuenta que este tipo de compuestos son menos utilizados en el área de estudio. Únicamente el pirimicarb se encontró en el 40% de las muestras analizadas, pero siempre en concentraciones inferiores al $0.1 \mu\text{g/L}$. El diazinon fue detectado en dos muestras pero únicamente en una de ellas la concentración sobrepasó el límite establecido por la legislación.

Por último y con el fin de verificar los resultados obtenidos, se decidió analizar simultáneamente algunas muestras utilizando cartuchos LiChrolut EN y Oasis HLB como sorbentes siguiendo el método de extracción SPE optimizado. Los resultados obtenidos (Tabla 4 de Anexos, página 132) mostraron que las concentraciones de pesticidas utilizando ambos sorbentes fueron similares y confirmaron la fiabilidad del método de extracción y análisis por GC-MS propuesto.

3.1.4.2. Evaluación de residuos de pesticidas en aguas mediante LC-MS

El método de análisis mediante GC-MS propuesto anteriormente para la evaluación de residuos de pesticidas en aguas permitió obtener resultados satisfactorios. Sin embargo para la evaluación posterior de un mayor número de pesticidas (58) (19 herbicidas, 18 fungicidas y 10 insecticidas) utilizados habitualmente en cultivos de La Rioja, especialmente en el cultivo de la vid, en un número más elevado de muestras de aguas (92) distribuidas en las tres zonas en la que se divide la Denominación de Origen de La Rioja se procedió a poner a punto un método analítico basado en LC-MS ya que se comprobó que algunos compuestos no se detectaban por GC-MS o se detectaban con mayor exactitud mediante LC-MS.

Para optimizar la detección por MS de todos los pesticidas seleccionados se inyectaron directamente en la fase móvil los patrones de cada pesticida ($10 \mu\text{g mL}^{-1}$) de forma individual, operando el aparato en modo scan. Las disoluciones fueron preparadas en metanol e inyectadas con la fuente de ionización de electrospray en modo positivo en un rango determinado de voltajes de cono entre 10 y 50 V y una velocidad de flujo de $15 \mu\text{g L}^{-1} \text{ min}^{-1}$.

Con el fin de obtener un método más sensible para la cuantificación de los pesticidas, se optó de nuevo por realizar una preconcentración previa al análisis usando el método SPE para alcanzar límites de detección inferiores al límite establecido por la legislación de $0.1 \mu\text{g L}^{-1}$. Se optimizaron los parámetros que pueden tener influencia en la eficiencia de la extracción como el tipo de sorbente del cartucho, el eluyente y el volumen de muestra. Se estudiaron cinco cartuchos de diferente naturaleza, tres contenían una fase polimérica (Oasis HLB, LiChrolut EN y Strata-X), uno contenía una base de sílice (C_{18}) y otro de carbón (SampliQ Carbon). Para calcular las recuperaciones se pasaron a través de cada tipo de cartucho 100 mL de agua ultrapura con una concentración de $1 \mu\text{g L}^{-1}$ de cada compuesto. La proporción más alta de recuperaciones ($>70\%$) se obtuvo con los cartuchos Oasis HLB (58% de los compuestos estudiados). Por esta razón los cartuchos Oasis HLB fueron seleccionados como los mejores sorbentes para la preconcentración de aguas. A su vez se ensayaron cinco disolventes para eluir los analitos retenidos en el cartucho: metanol, acetona, acetonitrilo, hexano y acetato de etilo. El mejor porcentaje de recuperación finalmente se obtuvo con una mezcla de 4 mL acetonitrilo + 4 mL de acetona. Con estas condiciones más del 90% de los analitos presentaban recuperaciones superiores al 65% (Figura 2 de Anexos, página 145).

El último paso de la optimización fue estudiar la influencia del volumen de muestra que se hace pasar por el cartucho. Se probó con volúmenes comprendidos entre 50 y 1000 mL, todos ellos con la misma concentración de cada analito ($0.2 \mu\text{g}$). En general, se observó que el volumen no influía en las recuperación de la mayoría de los analitos estudiados y únicamente para el pirimidinol, metamitron, metribuzina y los metabolitos hidroxilados de la atrazina se observaba una pequeña reducción de las recuperaciones cuando los volúmenes eran mayores de 250 mL y que esta disminución se acentuaba con volúmenes de 1000 mL. Finalmente se seleccionó un volumen de 500 mL ya que era el que ofrecía mejores resultados en cuanto a valores de recuperación y límites de detección más bajos que se pudieron obtener.

Con el fin de evaluar la influencia del efecto matriz en el análisis se realizó el protocolo de preconcentración anteriormente explicado con tres diferentes tipos de agua: ultrapura, de río y subterránea (exentas de pesticidas), previamente contaminadas con los analitos estudiados. Después de la preconcentración, las señales obtenidas para

los pesticidas en las muestras de agua superficial y subterránea se compararon con las obtenidas en las muestras de agua ultrapura. Así, se comprobó que en el agua subterránea el 63% de los compuestos estudiados registraban muy baja supresión o aumento de la señal (-15% a 15%) y el 23% de los compuestos presentaban una variación de la señal <25%. Se observaron efectos fuertes de matriz solamente para el 14% de los compuestos siendo mayor la influencia para los compuestos clorsulfuron, flazasulfuron, metribuzina y acetocloro. Se observaron resultados similares en las aguas superficiales, aunque el porcentaje de compuestos con muy bajo efecto fue del 68%. Por lo tanto, para compensar el efecto matriz y evitar una estimación errónea de los pesticidas, se decidió preparar patrones de calibración en agua subterránea (exenta de pesticidas) debido al gran número de muestras ambientales de esta clase.

Además, la validación del método se hizo para cada compuesto de forma individual, estudiando la exactitud y la precisión para el nivel de concentración establecido por la legislación de la Comunidad Europea y los límites de detección y cuantificación. Se prepararon ocho patrones dopados en la matriz con 500 mL de agua subterránea que contenían todos los analitos en un rango de concentración de 0.1 a 2 $\mu\text{g L}^{-1}$. Los resultados mostraron una buena relación lineal para todos los compuestos estudiados, con r^2 mayores de 0.99 para todos los compuestos en el rango estudiado. Únicamente seis compuestos presentaron límites de cuantificación mayores que el límite establecido por la UE para el agua potable (Tabla 3 de Anexos, página 146).

El método desarrollado y optimizado se aplicó para analizar 58 pesticidas en las muestras de aguas tomadas cercanas a zonas de cultivo de viñedo, detectándose al menos en una de las muestras 40 compuestos (6 insecticidas, 12 herbicidas, 16 fungicidas y 6 productos de degradación). Sin embargo algunos de los compuestos estudiados no se detectaron en ninguna de las muestras analizadas. Tanto los herbicidas terbutilazina, su metabolito desetilterbutilazina (DET) y fluometuron presentaron concentraciones altas en las muestras analizadas. Este hecho se debe a la frecuente aplicación de estos herbicidas en el área de estudio unido a que todos presentan unos valores del índice GUS entre 3.07 y 3.92, lo cual indica que estos compuestos pueden ser lixiviados fácilmente y llegar a las aguas. El incremento en la presencia de terbutilazina en agua fue debido a su uso en sustitución de atrazina, prohibida en Europa en el 2004 y finalmente retirada del mercado fitosanitario español en el 2007. Las altas

concentraciones detectadas de fluometuron podrían ser debidas al uso conjunto de este compuesto con la terbutilazina en formulaciones comerciales.

Otros herbicidas detectados en un gran número de muestras (72%) fueron etofumesato y diuron pero presentaron concentraciones bajas en la mayoría de las muestras, aunque en un porcentaje menor de muestras (29%) presentaron concentraciones más altas. La presencia de etofumesato se justifica al igual que para los compuestos anteriores por tener un índice de GUS muy alto (3.38), sin embargo el diuron presenta un índice de GUS muy bajo (1.83). La presencia de diuron podría ser debida al uso intensivo de este herbicida en el área y a la extensiva aplicación en la época del año en que fueron tomadas las muestras de aguas. Atrazina y propazina se detectaron en el 40% de las muestras, excediendo el límite de $0.1 \mu\text{g L}^{-1}$ solamente en cuatro muestras en cada caso. La presencia de estos compuestos, retirados del mercado hace varios años, en agua indica su alta persistencia. Igualmente, también se detectaron sus metabolitos, DEA y DIA en el 20% de las muestras confirmando el problema de contaminación ambiental debida al uso de estos herbicidas.

Los fungicidas detectados en un gran número de muestras de agua superficial y subterránea a diferentes concentraciones fueron pirimetanil, tebuconazol, kresoxim-metil, metalaxil y sus metabolitos (CGA-62826 y CGA-92370) y triadimenol. La presencia de este grupo de pesticidas, con un índice GUS entre 1.51 y 2.65, se debe a su uso intensivo en el cultivo de la vid y a su uso creciente en otros cultivos como los cultivos hortícolas, frutales y cereales.

Por último, los insecticidas fueron detectados en un menor número de muestras, ya que es poco frecuente su aplicación en las áreas estudiadas. Dentro de este grupo, los que más se detectaron fueron pirimicarb y diazinon (<40% de las muestras), dimetoato (<20% de las muestras) y cloropirifos y metoxifenocida (<5% de las muestras). Los compuestos que presentan una alta solubilidad como el dimetoato o un índice GUS elevados como metoxifenocida (3.02) fueron detectados en concentraciones superiores al límite establecido por la UE.

Finalmente se estudió la distribución espacial de las muestras de agua superficial o subterránea dentro de las tres sub-áreas en las que se divide la DOCa de La Rioja que presentaron concentraciones correspondientes a la suma de todos los compuestos

detectados mayores que $0.5 \mu\text{g L}^{-1}$ (límite establecido por la UE para el agua de consumo humano) (Tabla 4 de Anexos, página 153).

De las 75 muestras que corresponden a agua subterránea, 48 muestras distribuidas en la Rioja Alavesa, 20 en la Rioja Alta y 17 localizadas en la Rioja Baja mostraron valores de concentraciones correspondientes a la suma de compuestos detectados superiores a $0.5 \mu\text{g L}^{-1}$. Las 27 muestras restantes presentaron valores por debajo de este límite. Además se observó una cierta relación negativa, aunque no significativa, entre la profundidad del agua subterránea y el contenido total de pesticidas. Las concentraciones más altas correspondían en general a aguas de pozos de 1-3 m de profundidad en áreas donde la vid era el único cultivo, debido a las cantidades más altas de pesticidas aplicadas en viñedos para la protección del cultivo y las aguas subterráneas menos contaminadas se localizaban en áreas con otro tipo de cultivos como cereales o frutales. En las aguas superficiales la suma de las concentraciones de compuestos detectados fue mayor de $0.5 \mu\text{g L}^{-1}$ en el 62% de las muestras, 2 muestras en la zona de la Rioja Alavesa, 3 muestras en la Rioja Alta y otras 3 en la Rioja Baja.

A pesar de la alta cantidad de pesticidas detectados en las aguas subterráneas de La Rioja, se observó que la concentración total de pesticidas detectada a la entrada del río Ebro en La Rioja disminuye a la salida del río de la región. Posiblemente este resultado es debido al menor caudal del río en la entrada en la región que a su salida ya que existe una gran contribución de caudal aportada por sus afluentes en esta región y el consiguiente efecto de dilución.

3.1.5. Evaluación de residuos de pesticidas en suelos de zonas de viñedo de La Rioja

Se llevó a cabo una evaluación de residuos de pesticidas en suelos de zonas de viñedo de La Rioja próximos a las captaciones de aguas analizadas previamente. Se seleccionaron 17 pesticidas (8 fungicidas, 7 herbicidas y 2 insecticidas) y 3 productos de degradación entre aquellos detectados más frecuentemente en las muestras de aguas que habían presentado concentraciones superiores al límite establecido por la legislación Europea ($0.1 \mu\text{g L}^{-1}$). Inicialmente se procedió a optimizar un método de extracción y a validar un método multiresiduo de análisis de pesticidas en suelos. Posteriormente se aplicó el método optimizado a muestras reales de suelos de la región.

La detección de estos compuestos se llevó a cabo mediante los métodos LC-MS y GC-MS optimizados en el estudio de la evaluación de residuos de pesticidas en aguas. Pero debido a que el número de compuestos estudiados en los suelos fue menor que en las aguas fue necesario la modificación tanto de la rampa de temperaturas en el método de GC-MS, como de los gradientes de elución en el método LC-MS con el fin de optimizar los tiempos de análisis. Ambos métodos permitieron una buena separación y determinación de los pesticidas con una alta sensibilidad. Sin embargo, la determinación de todos los pesticidas sólo fue posible por LC-MS debido a que los productos de degradación HT y CGA-62826 y el insecticida metoxifenocida no se detectaron mediante GC-MS.

Inicialmente la optimización del proceso de extracción de los pesticidas requirió el estudio de la influencia del suelo en este proceso. Se contaminaron dos suelos con distinta textura y propiedades con una solución conteniendo todos los pesticidas en concentración adecuada correspondiente a una dosis de 0.1 mg kg^{-1} . Las extracciones se llevaron a cabo con diferentes disolventes de manera individual o con mezclas de disolventes. Se observó que los rendimientos en la extracción de los pesticidas no estaban afectados por el tipo de suelo posiblemente debido a que estos suelos presentaban una variación pequeña del contenido en materia orgánica, aunque el rango de variación era similar al que presentaban las muestras reales de suelo estudiadas. Sin embargo, se observaron diferencias significativas en las recuperaciones con los distintos disolventes (Figura S1 de Anexos, página 175), especialmente las recuperaciones de los pesticidas con baja solubilidad (kresoxim-metil y tebuconazol) disminuían drásticamente cuando se utilizaba agua como extractante. Cuando se utilizaron disolventes orgánicos de forma individual se obtuvieron recuperaciones, en general, $> 100 \%$ cuando los pesticidas se analizaron mediante LC-MS y variaron en el rango 60-120 % cuando se analizaron mediante GC-MS. Se encontraron excepciones para HT y CGA-62826 con recuperaciones $< 25\%$ para todos los extractantes y para pirimicarb con una recuperación $< 15\%$ con etil acetato. Al utilizar una mezcla metanol/acetona (50:50) las recuperaciones fueron $> 75 \%$ y $> 70 \%$ para todos los pesticidas excepto para HT y CGA-62826. En el caso de estos dos compuestos, las mejores recuperaciones (97 y 42 % respectivamente) se alcanzaron con una mezcla 50:50 de metanol/ CaCl_2 0.01 M o con una mezcla de metanol/agua (50:50) en un rango de pH entre 7 y 9 ($> 100 \%$).

Los ensayos planteados para estudiar la influencia del tiempo de envejecimiento de los pesticidas en el suelo en su recuperación indicaron que las recuperaciones no se vieron afectadas cuando el pesticida se aplicó a una dosis alta (1 mg kg^{-1}). Sin embargo, las recuperaciones disminuyeron al aumentar el tiempo de envejecimiento del pesticida en el suelo cuando se aplicaron a una dosis menor (0.1 mg kg^{-1}) (Tabla 3 de Anexos, página 167).

El efecto matriz fue también considerado como en el caso de las aguas en la determinación de los residuos de pesticidas. Para evitar este efecto se utilizó el extracto de un suelo no contaminado para preparar los patrones de la recta de calibración. El rango de concentraciones utilizado fue muy amplio ($0.005 - 1 \text{ } \mu\text{g mL}^{-1}$) con la finalidad de contemplar el rango de concentraciones obtenidas para algunos pesticidas en las muestras de suelo reales. Se determinaron los límites de detección y cuantificación de cada uno de los pesticidas obteniéndose valores menores de estos parámetros cuando los compuestos se determinaron por LC-MS que por GC-MS. Por lo tanto el análisis mediante LC-MS dio lugar a una mayor selectividad y precisión para la determinación de los pesticidas en los suelos (Tabla 4 de Anexos, página 169).

El método de análisis multiresiduo optimizado utilizando LC-MS se aplicó para la determinación de residuos de pesticidas en 17 muestras reales de suelos de viñedo de La Rioja. A su vez GC-MS se utilizó para la comprobación de los valores que pudieran resultar inciertos debido a su alta concentración, como en el caso de terbutilazina, fluometuron o acetocloro.

Una vez más, al igual que en los estudios previos de las aguas, el grupo de pesticidas más detectado fue el de los herbicidas, aunque las concentraciones en la mayoría de las muestras fueron menores que el límite de cuantificación. La terbutilazina y su metabolito DET se detectaron en todas las muestras. Únicamente la terbutilazina, su metabolito HT y el fluometuron alcanzaron concentraciones superiores a $90 \text{ } \mu\text{g kg}^{-1}$ en algunas muestras (Figura 2 de Anexos, página 170). Las altas concentraciones detectadas para estos herbicidas se deben al mayor uso de terbutilazina debido a la prohibición de la atrazina en 2004 y a la aplicación de formulaciones conteniendo terbutilazina y fluometuron. Los resultados obtenidos concuerdan con los resultados expuestos en los trabajos anteriores en los que terbutilazina y fluometuron fueron detectados en el 67% y 77% de las muestras de agua analizadas alcanzando

concentraciones de 12.6 y 18.7 $\mu\text{g L}^{-1}$ respectivamente. Sin embargo, el metabolito DET, muy presente en las muestras de agua analizadas, se detectó solamente en estos suelos a concentraciones por debajo del límite de cuantificación, mientras HT se detectó a concentraciones hasta 90.8 $\mu\text{g kg}^{-1}$. El acetocloro se detectó en concentraciones $> 5 \mu\text{g kg}^{-1}$ en algunas muestras de suelo.

Los fungicidas analizados se detectaron frecuentemente en los suelos con un porcentaje de muestras positivas por encima del 80%, excepto para kresoxim-metil y triadimol detectadas en un menor porcentaje de muestras, aunque con concentraciones superiores al LOQ para kresoxim. Un 41% de las muestras analizadas contenían el metabolito del metalaxil, CGA-62826, en concentraciones por encima del LOQ. Esto podría ser debido a la aplicación de metalaxil, presente en todas las muestras, y su rápida degradación para dar CGA-62826.

En el caso de los insecticidas, el pirimicarb se detectó en un mayor porcentaje de muestras que la metoxifenoza, un 76.5% de muestras positivas frente a un 58.8%, aunque fueron detectados por debajo del LOQ. Solamente en dos muestras la metoxifenoza se detectó a mayores concentraciones.

Se encontraron cantidades residuales en los suelos de los compuestos hidrofóbicos tales como HT, terbutilazina, kresoxim-metil, metoxifenocida y acetocloro con un $\log K_{ow} > 3$. Esto podría ser debido a la adsorción de estos compuestos por la materia orgánica (MO) del suelo, aunque no fue posible obtener una correlación positiva entre las cantidades residuales y el contenido en MO de los suelos debido al bajo número de muestras con concentraciones de pesticidas $> \text{LOQ}$.

3.2. USO DEL SUSTRATO POSTCULTIVO DE CHAMPIÑÓN COMO ESTRATEGIA FISICOQUÍMICA DE PREVENCIÓN Y/O CONTROL DE LA CONTAMINACIÓN.

3.2.1. Materiales

- **Suelos**

Los **suelos** utilizados pertenecen a viñedos de las tres sub-áreas de La Rioja (Figura 7) y se localizaron en **Alcanadre** (ALC) (42°24'20"N, 2°7'6"W), en La Rioja Baja, **Sajazarra** (SJZ) (42°35'18"N, 2°57'41"W) y **Yécora** (YEC) (42°32'47"N, 3°5'48"W) en La Rioja Alta. Se tomaron muestras de suelo del horizonte superficial (0-30 cm).

Las características fisicoquímicas de los tres suelos están detalladas en la Tabla 5. Estas se determinaron mediante los métodos de análisis de suelos establecidos (MAPA, 1986) en muestras tamizadas con un tamaño de malla < 2 mm. La composición granulométrica fue determinada por el método de la pipeta de Robinson. El carbono orgánico (OC) fue determinado mediante el procedimiento de oxidación del dicromato (Walkley-Black) y multiplicando los resultados obtenidos por 1.724 para obtener el contenido en materia orgánica (OM). El pH del suelo fue medido en una suspensión suelo:agua con una relación 1:1. El carbono inorgánico (CO_3^{2-}) fue determinado mediante el Calcímetro de Bernard. El nitrógeno total se determinó mediante el equipo Autoanalyzer 3 (Bran Luebbe) tras una digestión Kjeldhal.

La determinación del carbono orgánico soluble (DOC) se llevó a cabo en extractos de suelo con agua desionizada utilizando una relación suelo:agua 1:2 después de su agitación durante 24 h a $20 \pm 2^\circ\text{C}$ en cámara termostatzada. La suspensión fue separada por centrifugación a 10000 rpm (15 min) y posterior filtración. La determinación del carbono en solución se llevó a cabo por triplicado en un analizador de carbono orgánico total Shimadzu 5050 TOC-V_{CSH/CSN} (Shimadzu, Columbia, MD) utilizando rectas de calibrado de disoluciones patrón de ftalato ácido de potasio de diferentes concentraciones en el rango entre 1 y 1000 $\mu\text{M mL}^{-1}$.

La mineralogía de la fracción arcilla del suelo se determinó mediante la técnica de agregado orientado (Robert, 1975) a partir de una suspensión acuosa de la fracción arcilla del suelo (80 mg: 6 mL agua con hexametafosfato sódico al 2% como agente dispersante). Los diagramas de difracción de rayos X se obtuvieron en un difractómetro de rayos X Philips PW 1710 (Eindhoven, Holanda) utilizando la radiación $K\alpha$ del Cu y operando a 50 kV y 40 mA entre 3 y 60° a un tamaño de paso de 0.04° 2 θ y a una velocidad de paso de 2.5 s paso⁻¹. La identificación de los componentes minerales se estudió a partir de la posición de sus máximos de difracción con el software PCPDFWIN.

Tabla 5. Características de los suelos

| Suelo | Alcanadre | Sajazarra | Yécora |
|---------------------------------|----------------|------------------------|------------------|
| pH | 7.49 | 7.52 | 7.84 |
| OC % | 0.89 | 0.67 | 1.00 |
| DOC % | <0.01 | <0.01 | <0.01 |
| OM % | 1.53 | 1.15 | 1.72 |
| N % | 0.10 | 0.06 | 0.09 |
| C/N | 9.10 | 12.3 | 10.4 |
| CO ₃ ²⁻ % | 24.1 | 50.9 | 12.9 |
| Arena % | 79.6 | 67.0 | 34.6 |
| Limo % | 9.0 | 11.9 | 29.7 |
| Arcilla % | 11.4 | 21.1 | 35.7 |
| Mineralogía ^(*) | I, C, V | I, C | I, C, M |
| Textura | Franco arenoso | Franco arcillo arenoso | Franco arcilloso |

^(*)Ilita (I), Caolinita (C), Montmorillonita (M), Vermiculita (V).

En la Tabla 6 se incluye el análisis elemental para los tres suelos. Dicho análisis fue realizado mediante fluorescencia de rayos X (ED-XRF) con un espectrofotómetro SPECTRO XEPOS (AMETEK).

Tabla 6. Análisis elemental de los tres suelos seleccionados y del residuo postcultivo de champiñón

| Elemento | Alcanadre | Sajazarra | Yécora | SMS |
|---------------------------|-----------|-----------|--------|------|
| Mg (%) | 0.97 | 0.69 | 0.50 | 0.89 |
| Al (%) | 4.60 | 3.85 | 6.05 | 0.07 |
| Si (%) | 23.3 | 15.3 | 22.7 | 3.37 |
| P (%) | 0.06 | 0.03 | 0.04 | 0.60 |
| S (%) | 0.07 | 0.04 | 0.03 | 1.73 |
| Cl (%) | 0.05 | 0.02 | 0.02 | 0.07 |
| K (%) | 1.22 | 0.91 | 1.26 | 0.17 |
| Ca (%) | 7.73 | 15.8 | 3.21 | 6.01 |
| Ti (%) | 0.24 | 0.16 | 0.23 | 0.02 |
| V (mg kg ⁻¹) | 15.8 | 15.7 | 52.1 | 5.80 |
| Cr (mg kg ⁻¹) | 18.1 | 54.6 | 39.8 | 9.50 |
| Mn (mg kg ⁻¹) | 202 | 160 | 246 | 362 |
| Fe (mg kg ⁻¹) | 12380 | 9296 | 21790 | 2533 |
| Co (mg kg ⁻¹) | 50.2 | 55 | 43.0 | 3.0 |
| Ni (mg kg ⁻¹) | 47.2 | 15.5 | 25.3 | 8.80 |
| Cu (mg kg ⁻¹) | 52.3 | 74.6 | 45.5 | 92.3 |
| Zn (mg kg ⁻¹) | 1177 | 1301 | 1167 | 901 |
| Ba (mg kg ⁻¹) | 231 | 332 | 365 | 147 |
| Hg (mg kg ⁻¹) | 4.90 | 5.8 | 6.90 | <1.0 |
| Pb (mg kg ⁻¹) | 24.0 | 18.5 | 29.7 | <1.9 |

- **Residuo postcultivo de champiñón**

El residuo postcultivo del champiñón (SMS) procede del cultivo de *Agaricus bisporus* y fue suministrado por Sustratos de La Rioja S.L. (Pradejón). El SMS es una mezcla pasteurizada compuesta principalmente por paja de cereal, gallinaza, urea y yeso. El OC y DOC del SMS se determinaron siguiendo la misma metodología que para los suelos, pero en este caso se utilizó una relación residuo:agua 1:100. El OC fue 24.5% y el DOC 1.91%. El pH se midió en una suspensión del residuo en agua destilada en una proporción 1:2 (peso:volumen). Las cenizas se determinaron por diferencia de peso tras calcinación de 5 g de residuo a 540 °C durante 24 h. La humedad fue calculada por diferencia de peso tras secado de 5 g de SMS a 105 °C durante 24 h. El pH, el

contenido en cenizas y la humedad fueron 6.97, 33.6% y 64.5% respectivamente. Los resultados del análisis elemental del SMS se indican en la Tabla 6.

- **Pesticidas**

Se seleccionaron cuatro pesticidas con distintas características para los estudios llevados a cabo. En la Tabla 7 se incluyen las propiedades fisicoquímicas de los pesticidas estudiados. Estos compuestos se seleccionaron entre aquellos que se detectaron más frecuentemente en las aguas analizadas. Además se consideró para la selección sus diferentes características especialmente su carácter polar, siguiendo la clasificación establecida por Delle Site (2001), que considera un compuesto no polar cuando el log Kow es > 3.0 y móvil cuando log Koc es < 2.5 . Atendiendo a estos criterios: pirimicarb es considerado polar e inmóvil, tebuconazol es no polar e inmóvil, cimoxanilo es polar y móvil y por último, triadimenol es no polar y móvil (Figura 9).

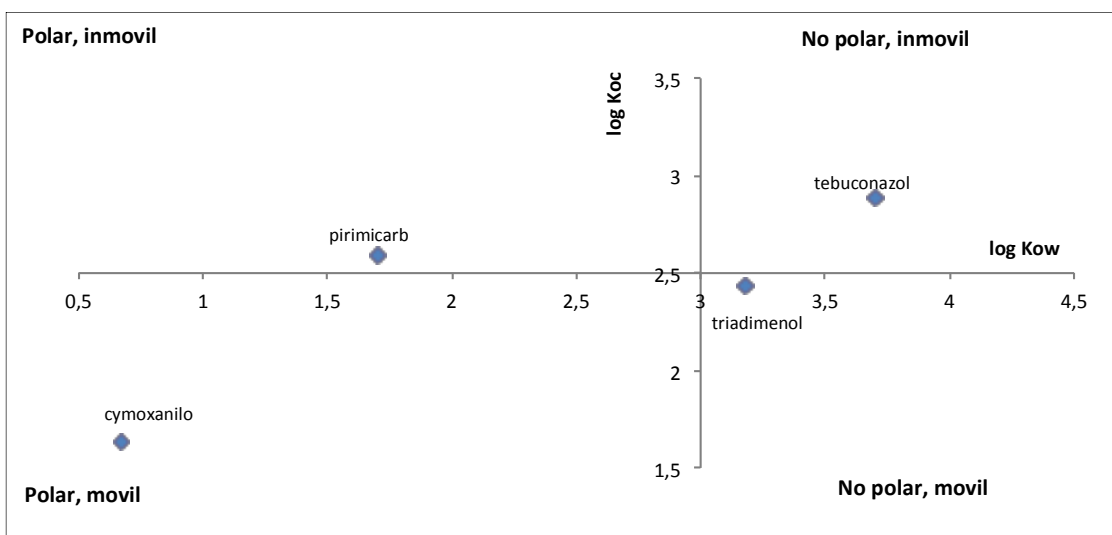
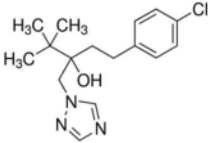
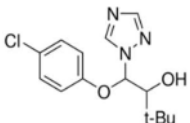
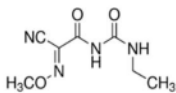
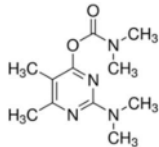


Figura 9 Clasificación de los pesticidas estudiados en función de su polaridad y movilidad.

Tabla 7. Estructura química y propiedades fisicoquímicas de los pesticidas estudiados

| Nombre común Nombre IUPAC | Tipo pesticida | Estructura química | PM (g mol ⁻¹) | Sol. Agua (mg L ⁻¹) | Log Kow | Koc (mL g ⁻¹) | DT ₅₀ (días ⁻¹) | Índice GUS |
|--|-------------------------------------|--|------------------------------|------------------------------------|------------|------------------------------|---|---------------|
| Tebuconazol | | | | | | | | |
| (<i>RS</i>)-1-p-clorofenil-4,4-dimetil-3-(1 <i>H</i> -1,2,4-triazol-1-ylmetil)pentan-3-ol | Fungicida (triazol) |  | 307.82 | 36 | 3.7 | 769 | 63-365 | 2.0 |
| Triadimenol | | | | | | | | |
| (1 <i>RS</i> ,2 <i>RS</i> ;1 <i>RS</i> ,2 <i>SR</i>)-1-(4-clorofenoxi)-3,3-dimetil-1-(1 <i>H</i> -1,2,4-triazol-1-yl)butan-2-ol | Fungicida (triazol) |  | 295.76 | 72 | 3.18 | 273 | 64.9-250 | 3.75 |
| Cymoxanilo | | | | | | | | |
| 1-[(<i>EZ</i>)-2-ciano-2-metoxiiminoacetil]-3-etilurea | Fungicida (cianoacetamida oxima) |  | 198.18 | 780 | 0.67 | 43.6 | 0.7-3.5 | -0.37 |
| Pirimicarb | | | | | | | | |
| 2-dimetilamino-5,6-dimetilpirimidin-4-yl dimetilcarbamato | Insecticida (carbamato) |  | 238.39 | 3100 | 1.7 | 388 | 86 | 2.73 |

Fuente PPDB, Pesticide Properties DataBase (2015). Sol. Agua, solubilidad en agua a 20 °C; Kow, coeficiente de partición octanol/agua a pH 7 y 20°C; Koc, coeficiente de adsorción normalizado al contenido de carbono orgánico, DT₅₀ tiempo de vida media de degradación aeróbica en suelo; Índice GUS, indicador de movilidad potencial.

3.2.2. Métodos Experimentales

- **Preparación de los suelos enmendados**

Los tres suelos seleccionados fueron enmendados con el sustrato postcultivo de champiñón (SMS) a distintas dosis (0%, 2%, 5%, 10%, 25%, 50% y 75% p/p). Las características de los suelos modificados determinadas como se ha indicado para los suelos no enmendados se incluyen en la Tabla 8. Los suelos sin enmendar y enmendados se colocaron en contenedores de 60 x 40 x 25 cm situados en invernadero y se mantuvieron en condiciones ambientales de temperatura, humedad y luz hasta su utilización en los estudios llevados a cabo (Figura 10).

Tabla 8. Características de los suelos enmendados

| Suelo (S) | Alcanadre | | | Sajazarra | | | Yécora | | |
|-----------|-----------|--------|---------|-----------|--------|---------|--------|--------|---------|
| | pH | OC (%) | DOC (%) | pH | OC (%) | DOC (%) | pH | OC (%) | DOC (%) |
| S+2%SMS | 7.39 | 1.22 | 0.056 | 7.40 | 0.97 | 0.042 | 7.70 | 1.78 | 0.038 |
| S+5%SMS | 7.26 | 2.22 | 0.062 | 7.26 | 1.73 | 0.062 | 7.46 | 2.22 | 0.047 |
| S+10%SMS | 7.22 | 4.00 | 0.277 | 7.25 | 3.56 | 0.199 | 7.56 | 4.89 | 0.169 |
| S+25%SMS | 7.15 | 7.11 | 0.342 | 7.21 | 6.25 | 0.399 | 7.36 | 9.33 | 0.454 |
| S+50%SMS | 7.10 | 15.6 | 0.446 | 7.19 | 16.3 | 0.439 | 7.29 | 12.2 | 0.442 |
| S+75%SMS | 7.06 | 18.9 | 1.063 | 7.09 | 17.6 | 1.211 | 7.59 | 19.3 | 0.840 |



Figura 10. Suelos no enmendados y enmendados con distintas dosis de SMS en condiciones ambientales

- **Determinación cuantitativa de los pesticidas**

En la Tabla 9 se indican las condiciones experimentales para la determinación de los pesticidas mediante LC-MS/DAD utilizando un equipo cromatográfico Waters (Waters Assoc., Milford, MA, USA).

Tabla 9. Condiciones cromatográficas establecidas para la detección de los pesticidas

| Condiciones | Tebuconazol | Triadimenol | Cimoxanilo | Pirimicarb |
|----------------------------------|---|---|----------------------------------|---|
| Fase móvil | 10:90 (H ₂ O+fórmico 0.1% : ACN) | 20:80 (H ₂ O+fórmico 0.1% : ACN) | 30:70 (H ₂ O: ACN) | 20:80 (H ₂ O+fórmico 0.1% : ACN) |
| Flujo (mL min ⁻¹) | 0.4 | 0.3 | 0.3 | 0.3 |
| Vol. inyec. (μL) | 20 | 20 | 10 | 20 |
| λ (nm)* | - | - | 253 | 244 |
| m/z | 308.80 | 296.76 | 199.19 | 239.29 |
| T _R (min) | 6.2 | 5.07 | 7.9 | 4.5 |
| Columna | Phenomenex Luna 3 μ C18, 150 x 4.60 mm | | | |

* Tebuconazol y triadimenol no se detectaron por PDA

- **Determinación cuantitativa de pesticidas marcados en ¹⁴C**

En los ensayos de movilidad y disipación se utilizaron compuestos marcados: ¹⁴C-tebuconazol y ¹⁴C-cimoxanilo. Ambos compuestos fueron suministrados por IZOTOP Co.Ltd. (Hungria). La actividad específica del ¹⁴C tebuconazol fue 4.72 MBq mg⁻¹ y la del ¹⁴C-cimoxanilo fue 10.08 MBq mg⁻¹, con una pureza radioquímica de 98.06% y 98.45% y una pureza química de 95.48% y 97.79%, respectivamente.

La cuantificación de estos compuestos se llevó a cabo mediante la técnica de centelleo líquido. Se añadió 4 mL de líquido de centelleo (Ecoscint TMA, National Diagnostics, Atlanta, GA) a 1 mL de la solución que contiene el pesticida marcado y se

midio la actividad en un contador de centelleo líquido Beckman LS6500 (Beckman Instruments, Inc., CA, USA).

3.2.2.1. Estudios de adsorción

El conocimiento del comportamiento de los pesticidas en el suelo es necesario a la hora de plantear estrategias para mitigar la contaminación. Uno de los procesos más importantes que afecta al comportamiento de los pesticidas en el suelo es el de adsorción ya que afecta en mayor o menor medida a los demás procesos que controlan el comportamiento de estos compuestos. Los factores del suelo con mayor influencia en el proceso de adsorción son el pH, el contenido en OM, la humedad y el contenido en arcilla. La concentración del pesticida también tiene un efecto en el proceso de adsorción, generalmente la adsorción aumenta al aumentar la concentración del pesticida en solución aunque en algunos casos no se observa influencia (Giles y col., 1960). En el sistema pesticida-suelo podemos cuantificar la adsorción del pesticida por el suelo obteniendo isothermas de adsorción, considerando el pesticida como adsorbato y el suelo como adsorbente, haciendo interaccionar cantidades conocidas de adsorbente con disoluciones con diferentes concentraciones iniciales de pesticida a una temperatura determinada.

La cantidad de pesticida adsorbida se determina, en condiciones de equilibrio, a partir de la diferencia entre la cantidad inicialmente presente en la solución (concentración inicial de pesticida, C_i) y la cantidad existente en la solución de equilibrio (concentración de equilibrio del pesticida, C_e) tras poner en contacto una cantidad determinada de adsorbente (m) y un volumen (V) de la disolución de adsorbato de concentración C_i . Para calcular la cantidad adsorbida (C_s) por unidad de masa de adsorbente se utiliza la siguiente expresión:

$$C_s = \frac{(C_i - C_e) \times V}{m}$$

De acuerdo con Giles y col. (1960), las isothermas de adsorción se pueden clasificar en cuatro tipos principales dependiendo de la pendiente del tramo inicial de la curva (Figura 11).

- ✓ *Isoterma tipo S*: La curvatura inicial de la isoterma indica que la adsorción es baja y se incrementa a elevadas concentraciones de soluto (adsorbato). Suele darse cuando hay una interacción específica entre el adsorbato y el adsorbente, cuando existe una moderada atracción entre las moléculas adsorbidas o cuando las moléculas de soluto y las de disolvente compiten por los sitios específicos de adsorción.

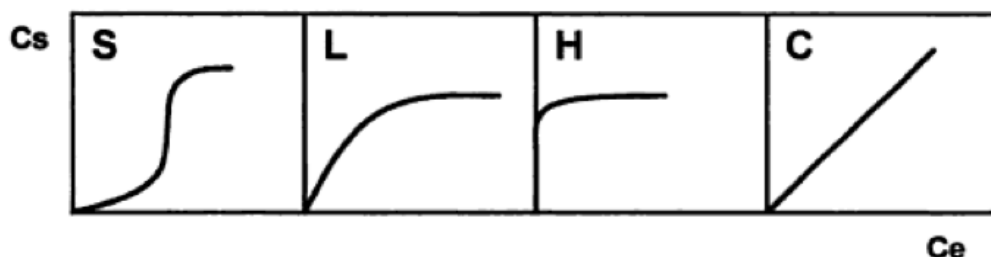


Figura 11. Clasificación de las isothermas de adsorción según Giles y col. (1960)

- ✓ *Isoterma tipo L*: la curvatura inicial de la isoterma indica que a la medida que transcurre la adsorción aumenta la dificultad para que las moléculas de soluto encuentren lugares de adsorción vacantes. En este caso no se dan fenómenos de competencia entre el soluto y el disolvente por los lugares de adsorción, hablando de adsorción específica.
- ✓ *Isoterma tipo H*: se ha clasificado como un caso especial dentro de la isoterma tipo L. Este tipo de isoterma se presenta cuando el adsorbente tiene una gran afinidad por el soluto, de modo que a bajas concentraciones se adsorbe todo y la concentración en el equilibrio será próxima a cero, una vez saturada la superficie del adsorbente, permaneciendo en solución el soluto en exceso.
- ✓ *Isotermas tipo C*: presentan una relación lineal entre la cantidad de soluto adsorbido y la concentración del mismo en la disolución de equilibrio. A este tipo de isoterma también se la conoce como de reparto constante.

Según Giles y col. (1960) la forma de la isoterma está relacionada con el mecanismo de adsorción, la naturaleza de la molécula a adsorber y de la superficie del adsorbente.

Para representar numéricamente las isothermas de adsorción de los pesticidas, éstas suelen ajustarse a ecuaciones matemáticas o modelos de adsorción, entre los cuales destacan la **ecuación de Freundlich** (1909) y la de **Langmuir** (1918), siendo el

modelo de Freundlich el más utilizado en la literatura. Esta es una ecuación empírica que relaciona la cantidad de soluto adsorbido con la concentración en equilibrio existente en disolución, y viene dada por la siguiente expresión:

$$C_s = K_f C_e^{n_f}$$

donde C_s ($\mu\text{g g}^{-1}$) es la cantidad de pesticida adsorbido por unidad de masa de adsorbente, C_e ($\mu\text{g mL}^{-1}$) es la concentración de equilibrio del pesticida en solución, y K_f ($\mu\text{g}^{1-n_f} \text{g}^{-1} \text{mL}^{n_f}$) y n_f son dos constantes características de la capacidad de adsorción del pesticida por el suelo. Los valores de estas dos constantes se determinan a través de la forma lineal de la ecuación:

$$\log C_s = \log K_f + n_f \log C_e$$

La constante n_f refleja la variación de la adsorción con la concentración (curvatura de la isoterma). Mientras, K_f es la cantidad de pesticida adsorbido para una concentración de equilibrio igual a la unidad ($C_e = 1 \mu\text{g mL}^{-1}$) y representa la adsorción a bajo nivel de concentración. Cuando se quiere conocer la adsorción a un nivel de concentración mayor, se calcula el coeficiente de distribución, K_d (mL g^{-1}), que mide la relación entre la concentración del pesticida en el suelo y en solución en condiciones de equilibrio para una concentración de equilibrio dada mediante la siguiente expresión:

$$K_d = \frac{C_s}{C_e}$$

- **Ensayo de adsorción**

El estudio de adsorción de los pesticidas por los suelos se llevó a cabo mediante la técnica de “batch” en el equilibrio. Para este estudio se utilizaron los tres suelos descritos anteriormente (Alcanadre, Sajazarra y Yécora) sin enmendar y enmendados con SMS a distintas dosis (2%, 5%, 10%, 25%, 50% y 75%) incubados durante un mes antes de la toma de muestras para el estudio de adsorción. Todas las muestras se tamizaron en el laboratorio ($< 2 \text{ mm}$). La adsorción de tebuconazol y cimoxanilo se llevó a cabo por los tres suelos, y se seleccionaron los suelos de Alcanadre y Sajazarra para el estudio de la adsorción de pirimicarb y triadimenol.

Las isothermas de adsorción de los pesticidas por los suelos se obtuvieron tratando 1 g de suelo con 10 ml de soluciones acuosas de cada pesticida con concentraciones iniciales de 1, 5, 10, 15, 20 y 25 $\mu\text{g mL}^{-1}$ para los compuestos no polares (tebuconazol y triadimenol) y de 1, 5, 10, 15, 20, 25, 50 y 100 $\mu\text{g mL}^{-1}$ para los polares (cimoxanilo y pirimicarb). Se utilizaron rangos de concentración diferentes de acuerdo con las solubilidades de los compuestos estudiados. También se prepararon muestras control (suelo sin pesticida y soluciones sin suelo) utilizadas como blancos. Para el estudio del tebuconazol y cimoxanilo se utilizaron los compuestos marcados con ^{14}C con una actividad de 100 Bq mL^{-1} . Las suspensiones se mantuvieron en contacto durante 24 h a $20\pm 2^\circ\text{C}$ en cámara termostatzada, con agitación intermitente. En experimentos preliminares se observó que 24 h era el tiempo suficiente para alcanzar el equilibrio. Posteriormente fueron centrifugadas a 5045 g durante 15 minutos. Todas las determinaciones se llevaron a cabo por duplicado.

Las concentraciones de equilibrio del pirimicarb y triadimenol fueron analizados mediante HPLC-MS con las condiciones descritas en la Tabla 9. Las concentraciones de ^{14}C -tebuconazol y ^{14}C -cimoxanilo en equilibrio se determinaron midiendo la actividad en un contador de centelleo líquido Beckman LS6500 (Beckman Instruments, Inc., CA, USA) como se ha indicado anteriormente.

La cantidad de pesticida adsorbida se calculó por diferencia entre la cantidad inicialmente presente en la solución y la obtenida en la solución de equilibrio. Estas isothermas se ajustaron a la ecuación de Freundlich. Los coeficientes de distribución K_d values fueron determinados para concentraciones de equilibrio $>1 \mu\text{g mL}^{-1}$ y se calcularon los coeficientes normalizados a 100% OC (K_{oc}) como $K_d 100\%OC$. Los valores de desviación estándar (SD) se calcularon para indicar la variabilidad entre réplicas. Para relacionar la adsorción con las características de cada suelo se utilizaron modelos de regresión lineal simple y múltiple usando el programa IBM SPSS (versión 22; USA).

3.2.2.2. Estudios de disipación

Los estudios de disipación de pesticidas en suelos se llevaron a cabo mediante la determinación a diferentes tiempos de la concentración remanente del compuesto después de un proceso de incubación del suelo-pesticida a temperatura y humedad

controlada en el laboratorio. La representación de dicha concentración, expresada habitualmente como porcentaje de la cantidad de pesticida aplicada inicialmente al suelo, frente al tiempo, permite determinar los parámetros de disipación del pesticida mediante el ajuste de los datos a ecuaciones cinéticas adecuadas. Los parámetros determinados habitualmente son la constante de velocidad de degradación (k) y tiempo de vida media (DT_{50}) o tiempo para que se disipe el 50% de la concentración inicial del pesticida en el suelo bajo unas condiciones específicas de incubación. Estos parámetros permiten estimar la persistencia de un compuesto en el suelo.

Uno de los modelos matemáticos utilizados para simular el comportamiento de pesticidas a partir de los resultados experimentales en el suelo es el **modelo cinético de primer orden (SFO)**. Este modelo está descrito por una ecuación exponencial simple con tan sólo dos parámetros. El modelo asume que el número de moléculas de pesticida es pequeño en relación al número de microorganismos y enzimas capaces de degradarlo. Como consecuencia, la velocidad de cambio en la concentración del pesticida (dC/dt) a cualquier tiempo es directamente proporcional a la concentración presente en el sistema. Para las cinéticas de primer orden, el tiempo para que la concentración disminuya proporcionalmente será constante a lo largo del experimento e independiente de la concentración de pesticida. Esta cinética de primer orden viene definida por la ecuación:

$$\frac{dC}{dt} = -kC$$

siendo su forma integrada:

$$C = C_0 \times e^{-kt}$$

donde C es la concentración del pesticida a un tiempo t , C_0 es la concentración inicial de pesticida a tiempo 0 y k es la velocidad de disipación. La vida media (DT_{50}) se calcula utilizando la siguiente ecuación:

$$DT_{50} = \frac{\ln 2}{k}$$

Como la degradación no siempre puede ser descrita por cinéticas de primer orden debido a que en muchos casos la degradación tiene un comportamiento bifásico, un descenso inicial rápido en las concentraciones de pesticida puede ir seguido por un

descenso más lento. Uno de los modelos que describe este comportamiento bifásico es el descrito por Gustafson y Holden (1990) o **modelo cinético de primer orden multi-compartimental (FOMC)**. Los autores definen el suelo como un medio heterogéneo espacialmente variable, sugiriendo que la degradación no ocurre a la misma velocidad dentro de los espacios individuales del sistema suelo. Dividieron el suelo en un gran número de sub-compartimentos sin conexión, cada uno de los cuales presenta una velocidad de degradación de primer orden. La distribución de estos coeficientes de velocidad da lugar a una ecuación relativamente simple con sólo tres parámetros, mostrando un comportamiento bifásico de degradación del pesticida en el suelo. Hay que mencionar que la ecuación inicialmente propuesta por Gustafson y Holden sufre una modificación en el modelo FOMC, el parámetro β del modelo FOMC corresponde al valor recíproco ($1 / \beta$) de la ecuación original. Siendo la ecuación final del modelo FOMC:

$$\frac{dC}{dt} = - \frac{\alpha}{\beta} C \left(\frac{t}{\beta} + 1 \right)^{-1}$$

y la forma integrada de la ecuación es :

$$C = \frac{C_0}{\left(\frac{t}{\beta} + 1 \right)^{\alpha}}$$

donde C es la concentración de pesticida a tiempo t, C_0 es la concentración inicial de pesticida a tiempo 0, α es un parámetro de forma determinado por el coeficiente de variación de los valores de las constantes de primer orden y β es un parámetro de localización. La vida media (DT_{50}) se calculó utilizando la ecuación:

$$DT_{50} = \beta(2^{1/\alpha} - 1)$$

Atendiendo a esta ecuación, la disipación es más rápida para valores mayores de α y valores pequeños de β . Sin embargo, el segundo término de la ecuación incluye el tiempo, de esta manera la velocidad de degradación dependerá del tiempo.

En algunos casos la cinética de degradación de pesticidas puede transcurrir de forma que la concentración inicial se mantenga constante durante un periodo de tiempo,

para luego producirse un descenso de la concentración de pesticida que pueda describirse por un modelo monofásico o bifásico. Esta fase inicial se denomina fase *lag* o de latencia y es debida al periodo de adaptación que sufren los microorganismos hasta que comienzan a degradar el pesticida (Rodríguez-Cruz et al., 2007). En este caso, el tiempo de vida media real del pesticida sería la suma de esta fase inicial y del valor de DT_{50} calculado para la segunda fase.

- **Ensayo de disipación**

El estudio de disipación de pesticidas se llevó a cabo en el suelo de Sajazarra, no enmendado y enmendado con dosis de SMS de 5% y 50% (p/p), como se ha explicado anteriormente. Tanto los suelos enmendados como los no enmendados fueron previamente incubados en condiciones ambientales de luz, temperatura y humedad durante un mes antes del estudio de disipación.

Los experimentos se llevaron a cabo por duplicado para conocer los mecanismos de disipación y biodisponibilidad de dos fungicidas seleccionados, tebuconazol y cimoxanilo, con diferentes características, y se utilizaron compuestos no marcados y marcados en ^{14}C .

Los fungicidas no marcados se aplicaron a 500g de suelo para obtener una concentración de 2 mg kg^{-1} . Las muestras se incubaron en una cámara termostatzada a $20^{\circ}C$ en oscuridad. La humedad fue ajustada con agua destilada a un 40% de la capacidad de campo del suelo. Con el fin de comprobar si se produce degradación química se preparó el mismo experimento con muestras esterilizadas de suelo para utilizarlas como control. Dos muestras de 500 g de suelo se esterilizaron en autoclave a $120^{\circ}C$ durante una hora, en tres días consecutivos. El ensayo de disipación con la muestra esterilizada del suelo siguió el mismo protocolo pero siempre se manipuló bajo condiciones estériles en una cabina de flujo laminar.

Se tomaron muestras de suelo a diferentes tiempos de incubación y se llevó a cabo la extracción del compuesto para la determinación de su contenido total. Muestras de 5 g de cada duplicado se agitaron durante 24 h en una cámara termostatzada ($20^{\circ}C$) con 10 mL de metanol en el caso del tebuconazol y con 10 mL de una mezcla 50:50 de metanol/agua en el caso del cimoxanilo. Posteriormente las suspensiones se

centrifugaron a 5045 g durante 15 minutos y los extractos resultantes fueron filtrados a través de filtros Minisart NY 25 (Sartorius Stedim Biotech, Alemania). Para la determinación cromatográfica de los fungicidas, 5 mL del filtrado fue llevado hasta sequedad mediante un evaporador con corriente de nitrógeno utilizando un evaporador EVA-EC2-L (VLM GmbH, Bielefeld, Alemania). El residuo se disolvió en 0.5 mL de acetonitrilo y fue transferido a un vial para el análisis. La determinación se realizó mediante HPLC-MS (véanse condiciones especificadas en la Tabla 9).

Además de la monitorización de la disipación, se llevaron a cabo estudios cualitativos de dos metabolitos del cimoxanilo: 2-cyano-2-methoxyiminoacetic acid ($m/z=128.09$) y 3-ethyl-4-(methoxyamino)-2,5-dioxoimidazolidine-4-carboxamide ($m/z=216.2$) y cuatro del tebuconazol: 3,3-dimethyl-1-([1,2,4]triazol-1-yl)-butan-2-one ($m/z: 168$), 5-tert-butyl-5-(1H-1,2,4-triazol-1-ylmethyl)hexanoic acid ($m/z=224$), 4-hydroxy-5,5-dimethyl-4-(1H-1,2,4-triazol-1-ylmethyl)hexanoic acid ($m/z=240$) y 1-(4-chlorophenyl)-3-hydroxy-4,4-dimethyl-3-([1,2,4]triazol-1-ylmethyl)-pentan-1-one ($m/z=322$).

De forma simultánea se llevaron a cabo experimentos con los pesticidas marcados en ^{14}C . Una alícuota del ^{14}C -pesticida se añadió al suelo no enmendado y enmendado (500 g) para obtener una concentración final de 2 mg kg^{-1} y una actividad de 100 Bq g^{-1} . Los suelos se incubaron a 20°C en la oscuridad ajustándose previamente la humedad al 40% de la capacidad de campo. En todas las muestras se colocó un vial con 1 mL de NaOH (1M), tal como indica Reid y col. (2002) con el fin de retener y cuantificar la fracción del pesticida mineralizada ($^{14}\text{CO}_2$). A diferentes tiempos de incubación se determinaron las fracciones de ^{14}C extraídas con CaCl_2 , metanol y la fracción no extraíble. Inicialmente 5 g de cada tratamiento se agitaron durante 24 h con 10 mL de una solución acuosa de CaCl_2 (0.01 M), seguida de una segunda extracción (24 h) con 10 mL de metanol. La cuantificación del ^{14}C -tebuconazol y ^{14}C -cimoxanilo se llevó a cabo mediante centelleo líquido en un contador Beckman LS6500 (Beckman Instruments Inc., CA), como se ha indicado anteriormente.

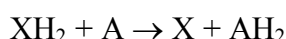
Los residuos de pesticida enlazado en el suelo después de las extracciones se cuantificaron mediante la combustión en un horno de combustión Biological Oxidizer OX500 (R.J. Harvey Instrument Corporation, Tappan, NY, USA) de 1 g de la muestra (por triplicado) de suelo seco bajo corriente de O_2 a 900°C . El $^{14}\text{CO}_2$ generado se

recogió en una mezcla 1:15 de etanolamina y líquido de centelleo (Oxisolve C-400, Zinsser Analytic). El $^{14}\text{CO}_2$ generado durante la incubación y retenido en el vial que contiene 1 mL de NaOH (1 M) se cuantificó añadiendo 4 mL del líquido de centelleo al vial como se ha indicado anteriormente.

Las cinéticas de degradación fueron ajustadas al modelo cinético de primer orden (SFO) y al modelo cinético de primer orden multi-compartmental (FOMC), explicados anteriormente. El análisis de varianza (ANOVA) se utilizó para evaluar los efectos de los tratamientos sobre la disipación. La desviación estándar (SD) se utilizó para indicar la variabilidad entre réplicas y la diferencia menos significativa (LSD), a un nivel de confianza del 95%, se determinó para evaluar los efectos de los diferentes tratamientos del suelo sobre los valores de DT50 y la actividad deshidrogenasa. Para los análisis de los datos se utilizó el software estadístico Statgraphics Plus versión 5.1 (Statgraphics Plus Corp., Princenton, NJ, USA).

- **Actividad deshidrogenasa**

La actividad deshidrogenasa (DHA), enzima asociada a la actividad oxidativa de los microorganismos sobre la materia orgánica, se considera un indicador del funcionamiento de las comunidades microbianas del suelo. La determinación de esta actividad comprende distintos sistemas de enzimas, las cuales intervienen en procesos de deshidrogenación, representados por la siguiente reacción (Paolini, 2003):



Donde XH_2 es un compuesto orgánico (donador de hidrógenos) y A es el aceptor de hidrógenos.

Para la determinación de la actividad deshidrogenasa (DHA) se siguió el método de Tabatabai (Tabatabai, 1994). El cual se basa en el uso de una sal soluble, en este caso cloruro de 2,3,5-trifeniltetrazolium (TTC) al 3 %, como aceptor terminal de electrones. Para ello se tomaron por duplicado 6 g de muestra y se mezclaron con 0.06 g de CaCO_3 , 1 mL de la disolución acuosa de TTC al 3% y 2.5 mL de agua ultrapura Milli-Q (Millipore). Después de incubar las muestras de suelo durante 24 h a 37 °C, esta sal es reducida formando 1,3,5-trifenilformazan (TPF) de color rojo. Una vez extraído el TPF con metanol, su concentración fue cuantificada por espectrofotometría UV-visible a una

longitud de onda de 485 nm en un espectrofotómetro Cary 100 Conc (Varian Optical spectroscopy Instruments) con un software Cary win UV.

3.2.2.3. Estudios de movilidad

El estudio de movilidad de pesticidas en suelos, una vez aplicados, puede llevarse a cabo, en experimentos de campo, a partir de la determinación de la concentración del compuesto en el flujo de agua recogido en lisímetros instalados en el campo tras un proceso de irrigación o un evento de lluvia o en el laboratorio utilizando columnas de suelo, ya sean empaquetadas manualmente o tomadas directamente en el campo “sin alterar”. Estas columnas son posteriormente sometidas a un flujo continuo o discontinuo de agua o solución acuosa de CaCl_2 0.01M, tras la aplicación del pesticida en la parte superior de la columna y determinando posteriormente la concentración del pesticida lixiviado por el extremo inferior de las mismas. Las curvas de lixiviación del pesticida se obtienen a partir de la determinación cuantitativa del pesticida en los volúmenes de eluyente obtenidos. Se representa la concentración del pesticida obtenida o el porcentaje de compuesto obtenido respecto al volumen total aplicado frente al volumen de eluyente recogido expresado en mL o en volúmenes de poro de la columna.

Un parámetro que se determina en los estudios de movilidad es el factor de retardo del pesticida (R_f). Este parámetro se determina como indicador del desplazamiento o variación del pico máximo de elución del pesticida observado en las curvas de lixiviación con respecto al que presenta el ión trazador y que describe el movimiento del agua en el suelo sin presentar ningún tipo de retención ni degradación del mismo. El factor de retardo teórico se calcula, siempre que sea lineal la isoterma de adsorción, de acuerdo con la expresión propuesta por Vincent y col. (2007):

$$R_f = 1 + \frac{K_f \rho}{\theta}$$

donde R_f es el factor de retardo, ρ es la densidad del suelo (g cm^{-3}), θ es el contenido volumétrico de agua y K_f es la constante de adsorción del pesticida en estudio por el suelo.

- **Ensayo de movilidad**

El suelo usado en este estudio fue el de Sajazarra, cuya textura es franco arcillo arenosa. Los ensayos de movilidad se llevaron a cabo en columnas empaquetadas de suelo de 3 cm (d.i) x 25 cm (longitud). Se utilizaron 100 g de suelo para el suelo no enmendado y enmendado con SMS al 5% y 80 g para el suelo enmendado con SMS al 50% y las columnas fueron saturadas con agua, previamente a su utilización, hasta alcanzar una humedad equivalente a la capacidad de campo del suelo para eliminar el aire ocluido en los poros del suelo. El volumen de poro de las columnas empaquetadas se calculó como la diferencia de peso entre el suelo saturado con agua y el suelo seco. Posteriormente se aplicó un volumen de 1 mL del pesticida marcado con ^{14}C disuelto en metanol (concentración 1 mg mL^{-1} y actividad $\approx 8333.3 \text{ Bq mL}^{-1}$) en la parte superior de las columnas. Cada experimento se realizó por duplicado.

Las curvas de percolación de los pesticidas se determinaron aplicando un volumen de 500 mL de solución de CaCl_2 0.01M a la columna en régimen saturado con una velocidad de 1 mL min^{-1} (aplicación continua) con una bomba peristáltica Gilson Miniplus 3 (Gilson, Inc., Middleton, WI, USA) y en régimen saturado-no saturado (25 mL durante 20 días) a la misma velocidad. Se recogieron fracciones de la solución lixiviada (15 mL) con un colector de fracciones automático Gilson F203.

El estudio de movilidad se llevó a cabo después de 24 horas de la aplicación del pesticida en la columna de suelo (no incubación) y después de 1 mes de su aplicación en la columna (incubación de las columnas con pesticida en condiciones controladas de temperatura a 20°C). Mineralización de los fungicidas durante el tiempo de incubación en las columnas o durante la aplicación del flujo saturado-no saturado fue también controlada mediante una trampa de $^{14}\text{CO}_2$ consistente en un vial con 1 mL de solución de NaOH 1M, colocada en la parte superior de la columna. La cuantificación de los pesticidas marcados con ^{14}C en las fracciones lixiviadas se realizó en un contador de centelleo líquido Beckman LS6500 (Beckman Instruments, Inc., CA, USA) como se ha indicado anteriormente.

Una vez terminado el periodo de lavado, las columnas de suelo se dividieron en tres tramos (0-5 cm, 5-10 cm y 10-15 cm) con el fin de realizar un balance total del compuesto. El suelo se secó a temperatura ambiente, tomándose muestras de 1 g de

suelo (por duplicado) correspondiente a cada tramo de las columnas. El ^{14}C -pesticida no lixiviado y enlazado al suelo se determinó por combustión del suelo en un horno de combustión como se ha explicado anteriormente.

Las curvas de lixiviación de un ión no interactivo fueron también obtenidas para determinar las características de dispersión de la columna y comparar los efectos con los pesticidas. Se aplicó a las columnas una cantidad de 47 mg ión cloruro por columna desde una solución de KCl y las curvas de lixiviación del **ión trazador cloruro** fueron obtenidas en condiciones similares a aquellas posteriormente obtenidas para los pesticidas aplicados.

El sistema cromatográfico utilizado fue un sistema modular de cromatografía iónica Metrohm (Metrohm Ltd., Herisau, Suiza) con supresión química formado por un módulo de bombeo para el eluyente (Metrohm 709 IC Pump), una bomba peristáltica para el bombeo de las disoluciones de lavado y regeneración del supresor (Metrohm 752 Pump Unit), el centro de separación donde se encuentra la columna y el módulo supresor (Metrohm 733 IC Separation Center), y el detector de conductividad (Metrohm 732 IC Detector). El sistema de adquisición y procesamiento de datos utilizado fue el IC Metrodata 714 para Win 95. La columna utilizada fue 6.1006.520 Metrosep A Supp 5 (Metrohm), constituida por alcohol polivinílico con grupos amonio cuaternario, de 150 mm x 4 mm d.i., de 5 μm de tamaño de partícula. La fase móvil fue una disolución en agua ultrapura Mili-Q (Millipore) con 3.2 mmol L^{-1} de Na_2CO_3 , 1.0 mmol L^{-1} de NaHCO_3 y 100 mL de acetona (al menos un 10% para evitar bacterias). Como soluciones de lavado y de regeneración del supresor se utilizaron agua ultrapura y una disolución acuosa de ácido sulfúrico 20 mM. La velocidad de flujo fue 0.6 mL min^{-1} y el volumen de inyección 100 μL . Antes de llenar el bucle de muestras manualmente (con jeringa), las muestras se filtraron con filtros GHP acrodiscs (Waters Corporation) de 0.2 μm . Con estas condiciones el tiempo de retención del ión trazador fue de 8.2 min.

3.2.3. Estudio de adsorción de pesticidas por suelos de viñedo de La Rioja no enmendados y enmendados con residuo postcultivo de champiñón

El objetivo de este trabajo fue estudiar la capacidad del SMS, utilizado como enmienda, como adsorbente de dos pesticidas no polares (tebuconazol y triadimenol) y dos polares (cimoxanilo y pirimicarb) con el fin de desarrollar una estrategia o método

fisicoquímico de inmovilización de pesticidas para limitar el transporte de estos compuestos y prevenir la contaminación de aguas. La adsorción de estos compuesto se llevó a cabo en tres suelos enmendados con SMS a diferentes dosis, ente 2% y 100%.

Las isothermas obtenidas para los dos pesticidas no polares estudiados (Figura 1 de Anexos, página 182) se ajustaron a la ecuación de Freundlich. Estas isothermas fueron no lineales de tipo L para la adsorción de ambos pesticidas por el suelo enmendado con diferentes dosis de SMS indicando una disminución de la adsorción a altas concentraciones del pesticida en solución. Sin embargo presentan un aumento de linealidad para los suelos enmendados con la dosis más elevada de SMS indicando mecanismos de adsorción o partición según la dosis de enmienda del suelo.

La adición de la enmienda al suelo dio lugar a un aumento del contenido en OC del mismo aumentando la adsorción de los dos pesticidas, siendo más significativo el incremento de la adsorción en el caso del tebuconazol. De acuerdo con esto se observó una correlación positiva para los dos pesticidas entre el coeficiente de adsorción K_f y el OC ($p < 0.01$). También fue positiva la correlación encontrada entre K_f y DOC ($p < 0.01-0.05$) probablemente derivada de la correlación positiva entre el OC y el DOC. Se puso de manifiesto que el DOC favorecía la adsorción a pesar de que algunos trabajos en la literatura han puesto de manifiesto la influencia negativa del DOC en la adsorción de pesticidas por los suelos. Posiblemente en este caso tuvo lugar la adsorción del DOC por el suelo con formación de nuevas superficies que favorecieron la adsorción de los pesticidas.

Para comparar la capacidad de adsorción de los pesticidas por los suelos enmendados con las diferentes dosis de enmienda se determinaron los coeficientes de distribución (K_d) a una concentración de equilibrio de $5 \mu\text{g mL}^{-1}$ (K_{d5}), debido a que los valores del parámetro n_f fueron diferentes de la unidad. Los valores K_{d5} aumentaron tras la aplicación de la enmienda (Tabla 3 de Anexos, página 183). Los tres suelos estudiados presentaron capacidades similares de adsorción, dependiendo aparentemente del contenido total de OC en la mezcla final SMS-suelo. De hecho los valores de K_{d5} normalizados respecto al OC variaron en un margen estrecho.

Sin embargo cabe señalar que la eficiencia del SMS aplicado al suelo para aumentar la capacidad de adsorción de los pesticidas dependía de la capacidad de

adsorción de los pesticidas por los suelos no enmendados (Figura 2 de Anexos, página 184). La eficiencia menor del SMS para la retención de los pesticidas se observó en el suelo YEC para tebuconazol y en el suelo SJZ para triadimenol debido a la mayor adsorción de los pesticidas por estos suelos. Posiblemente esto fue debido al contenido más alto en arcilla de ambos suelos o la presencia de montmorillonita en el suelo YEC, un mineral de la arcilla con gran capacidad de adsorción.

Los resultados indicaron que un aumento en el coeficiente de adsorción K_d de hasta 3-5 veces podría conseguirse para ambos compuestos cuando se aplique al suelo una dosis de SMS <10% que corresponde aproximadamente a una aplicación de 25-30 t C ha⁻¹ equivalente a una aplicación agronómica de SMS como una enmienda orgánica, Sin embargo una aplicación de una dosis de residuo más elevada podría ser usada cuando la inmovilización del pesticida fuera el objetivo con el fin de evitar la contaminación de las aguas desde una fuente puntual de contaminación. En este caso un aumento de K_d de más de 20 veces podría conseguirse.

El efecto del OC derivado del SMS en la retención del tebuconazol y triadimenol fue corroborado estadísticamente por la correlación significativa encontrada entre K_d y OC ($p < 0.001$). Los resultados obtenidos mediante un análisis de regresión múltiple incluyendo el parámetro DOC y el contenido en arcillas no afectaban significativamente en la capacidad de adsorción. Sin embargo, la inclusión del coeficiente K_{ow} de cada pesticida y el contenido en OC de los suelos en el modelo de correlación múltiple llegó a explicar más del 94% de la variabilidad del coeficiente de adsorción. La ecuación obtenida puede ser utilizada para predecir la adsorción de pesticidas no polares cuando se aplican diferentes dosis de SMS.

Las isothermas de adsorción de cimoxanilo por los suelos no enmendados y enmendados con diferentes dosis de SMS se ajustaron también a la ecuación de Freundlich, presentando isothermas no lineales tipo L (Figura 1 de Anexos página 182) aunque no se observaron cambios en la linealidad al aumentar la dosis de enmienda aplicada. Las isothermas de adsorción de pirimicarb presentaron la misma tendencia no lineal pero con isothermas tipo L y S, independientemente del suelo y la enmienda aplicada.

Los coeficientes de adsorción de los pesticidas polares fueron menores, entre 5 y 10 veces, que los obtenidos para los no polares. La adsorción se vio favorecida con el incremento del contenido en OC proveniente de la enmienda, pero se obtuvo una correlación entre K_f y OC con valores de r^2 menores que para los pesticidas no polares. Además la correlación entre K_f y DOM no fue significativa, lo que puede ser debido a que las moléculas disueltas compiten por los mismos lugares de adsorción que los pesticidas polares.

Igual que para los pesticidas no polares se determinaron los coeficientes de distribución para comparar los coeficientes de adsorción de los pesticidas por los suelos enmendados a diferentes dosis. En este caso se calcularon a una concentración de equilibrio de $40 \mu\text{g mL}^{-1}$ (K_{d40}). Estos coeficientes aumentaron, en general, para ambos pesticidas al aumentar la dosis de enmienda de los suelos (Tabla 4 de Anexos, página 186). El incremento de la adsorción fue menos pronunciado para el pirimicarb que para el cimoxanilo. Además, cuando la adsorción se llevó a cabo en el suelo de Sajazarra enmendado con dosis bajas de SMS la adsorción disminuyó en comparación con el suelo no enmendado.

Los resultados indicaron que un aumento del coeficiente de adsorción hasta 15 veces para cimoxanilo y hasta 4 veces para pirimicarb podría conseguirse cuando una dosis de enmienda $<10\%$ se aplicó en los suelos y aumentos más elevados en la adsorción de cimoxanilo podría conseguirse para dosis de SMS $>25\%$. Sin embargo un aumento en la adsorción de pirimicarb no se observó en todos los suelos indicando que el efecto del SMS no se puede generalizar para todos los suelos en el caso de la adsorción de pesticidas polares.

Aunque en general la adsorción de los compuestos polares aumentó con el contenido en OC proveniente del SMS, los valores de K_{d40} normalizados con OC indicaron que la adsorción de los pesticidas por el suelo enmendado se ve afectada en mayor medida por otras propiedades del suelo. La correlación entre K_{d40} y OC presentó un coeficiente r^2 menor que en el caso de los pesticidas no polares. No obstante, este coeficiente mejora cuando se tiene en cuenta las variables: DOC, K_{ow} y arcillas. A pesar de que para los pesticidas polares no se puede realizar una predicción con la misma confianza que para los pesticidas no polares, el incremento de la adsorción con la enmienda fue relevante a pesar de que son contaminantes hidrofílicos.

Finalmente con el fin de conocer la posibilidad de predecir los valores del coeficiente de adsorción K_d para pesticidas polares y no polares en suelos enmendados con SMS, se compararon los valores estimados y los experimentales tanto de los pesticidas no polares, tebuconazol y triadimenol, como de los polares, cimoxanilo y pirimicarb, en los suelos enmendados con distintas dosis. Los valores estimados de K_d se calcularon por la ecuación: $K_d = f_{sms} K_{d_{sms}} + f_{soil} K_{d_{soil}}$. Posteriormente se obtuvieron las regresiones lineales entre los valores de K_d estimados y experimentales para cada pesticida en cada tipo de suelo estudiado (Figura 3 de Anexos página 188). Los pesticidas no polares presentaron un grado de correlación mayor que los polares. Por tanto, en el caso de los no polares la estimación del parámetro K_d podría utilizarse para predecir la adsorción de estos compuestos en suelos enmendados con distintas dosis de SMS.

3.2.4. Estudio de la disipación de pesticidas en suelos de viñedo de La Rioja no enmendados y enmendados con residuo postcultivo de champiñón

En este estudio se evaluó el impacto de la enmienda (SMS) aplicada al suelo en dosis muy diferentes (5 y 50%) sobre la biodisponibilidad y disipación de dos fungicidas con el fin de conocer la posible evolución de estos compuestos adsorbidos por los suelos enmendados utilizados para desarrollar posibles estrategias de control o prevención de fuentes difusas o puntuales de contaminación.

Dos modelos cinéticos SFO y FOMC fueron aplicados para describir la cinética de disipación de ambos pesticidas en los suelos sin enmendar y enmendados. Cimoxanilo presentó un mejor ajuste al modelo SFO (Figura 1 de Anexos, página 199). Este compuesto presentó una disipación muy rápida (< 1 día) en todos los suelos estudiados. Los valores de DT_{50} comprendieron un rango de valores entre 0.4 y 0.8 días. La velocidad de disipación de este fungicida se incrementó cuando la enmienda fue añadida al suelo. En consecuencia no se observó la influencia de la adsorción (mayor en suelos enmendados) para disminuir la biodisponibilidad para la biodegradación de este compuesto y tampoco fueron significativos los cambios en la velocidad de degradación en los suelos enmendados con las dos dosis de SMS.

En el caso del tebuconazol la cinética se ajustó al modelo FOMC o SFO (Figura 1 de Anexos, página 199) cuando se aplicó al suelo no enmendado y enmendado al 5%,

o enmendado al 50%, respectivamente. El tebuconazol presentó valores de DT_{50} mayores que el cimoxanilo. También aumentó la velocidad de disipación en los suelos enmendados disminuyendo los valores de DT_{50} hasta casi 300 veces respecto a los obtenidos en el suelo no enmendado sin que la dosis de SMS tuviera efectos relevantes. Los coeficientes de adsorción de tebuconazol por los suelos no enmendados y enmendados no podrían explicar las cantidades más elevadas de tebuconazol al final del periodo de incubación en el suelo no enmendado.

Este hecho podría ser explicado considerando que la aplicación del SMS produzca un incremento de la actividad microbiana del suelo, favoreciendo la biodegradación del pesticida. A su vez la materia orgánica de la enmienda podría favorecer la adsorción del pesticida formando residuos enlazados, lo cual explicaría que se detectase una aparente disipación más rápida en suelos enmendados, como ha sido observado para otros compuestos en la literatura.

A lo largo del periodo de incubación se detectaron algunos metabolitos del tebuconazol hasta los 303 días a pesar de que experimentos de disipación de los fungicidas en los suelos esterilizados fue más lenta, indicando la influencia de la comunidad microbiana en el proceso. La formación de los metabolitos fue mayor en el suelo con menor dosis de enmienda, debido a que a dosis altas de enmienda se favorece la retención del pesticida por el suelo (Figura 2 de Anexos, página 201).

Con el objeto de determinar los mecanismos de disipación de ambos compuestos y su biodisponibilidad se determinó el balance de masa total a lo largo del proceso de disipación de ambos compuestos a partir de los porcentajes de ^{14}C determinados en las fracciones mineralizadas, extraídas con $CaCl_2$, extraídas con metanol y correspondientes a los residuos enlazados (Figura 3, de Anexos, página 203). Los resultados indicaron porcentajes entre 86 y 112% para los balances de masa total, expresados como porcentaje del ^{14}C inicial para tebuconazol y cimoxanilo en el suelo no enmendado y enmendados con el 5% y 50%.

La mineralización del cimoxanilo fue muy rápida al principio aunque la evolución del $^{14}CO_2$ fue progresiva en todos los suelos durante todo el periodo de incubación. ^{14}C -Cimoxanilo estaba marcado en el grupo acetilo indicando la evolución rápida del $^{14}CO_2$ debido a que este grupo se degrada rápidamente. La mineralización de

cimoxanilo fue siempre mucho más rápida que la de tebuconazol, correspondiéndose estos resultados con los obtenidos en la disipación global. ^{14}C -tebuconazol estaba marcado en el anillo triazol indicando la lenta mineralización de este grupo que debe ser menos accesible a los microorganismos. Para los dos fungicidas la mineralización siempre fue más alta en caso del suelo no enmendado. La mayor adsorción determinada en los suelos enmendados, explicada anteriormente, corroboraría estos resultados.

Las fracciones de tebuconazol extraídas con CaCl_2 fueron mayores en el suelo no enmendado, seguidas por el enmendado con el 5% y por último el enmendado con el 50% indicando el efecto de la dosis de SMS en el suelo en la extractabilidad del compuesto en agua y por tanto en su potencial biodisponibilidad. Estos resultados están relacionados con los coeficientes de adsorción del tebuconazol, siendo mayores en los suelos enmendados, indicando una mayor biodisponibilidad del fungicida en el suelo no enmendado. Sin embargo, las cantidades extraídas con metanol siguen el orden contrario, pero disminuyen con el tiempo en los suelos enmendados, de acuerdo con la mayor velocidad de disipación del fungicida en los suelos enmendados.

La fracción de cimoxanilo extraída con CaCl_2 fue inicialmente mayor que la del tebuconazol, de acuerdo con su potencial biodisponibilidad, y disminuyeron a lo largo del periodo de incubación. Las cantidades extraídas fueron mayores en el suelo no enmendado que en el suelo enmendado y disminuyeron en el suelo con mayor dosis de enmienda aplicada. Las fracciones extraídas con metanol fueron inicialmente más altas para los suelos enmendados y disminuyeron rápidamente en los suelos enmendados siendo $<1\%$ después de 9 días. Tanto las fracciones extraídas con CaCl_2 como aquellas extraídas con metanol deben corresponder a cimoxanilo y a los metabolitos del cimoxanilo formados durante su degradación de acuerdo con el porcentaje más elevado de ^{14}C -cimoxanilo extraído comparado con el porcentaje obtenido de cimoxanilo no marcado en el ensayo de disipación.

Las fracciones correspondientes a residuos no extraídos fueron inicialmente menores para el tebuconazol que para el cimoxanilo aumentando su formación a lo largo del periodo de incubación en el caso del tebuconazol, y disminuyendo para el cimoxanilo debido en este caso al aumento del $^{14}\text{CO}_2$ producido. Trabajos en la literatura han puesto de manifiesto que los residuos enlazados podrían estar disponibles para la degradación o mineralización debido a un re-equilibrio entre las fases del

compuesto adsorbido y soluble. Este hecho podría explicar los resultados obtenidos para el cimoxanilo, sin embargo la formación de residuos enlazados de tebuconazol continuó durante todo el periodo de incubación indicando una continua formación de residuos nuevos e interacciones fisicoquímicas potenciadas entre el fungicida y el suelo.

Los porcentajes correspondientes a los residuos enlazados del cimoxanilo incrementaron con la dosis alta de enmienda, siendo similares para el suelo no enmendado y enmendado con la dosis más baja. Para el tebuconazol los porcentajes más altos se observaron en los suelos enmendados con ambas dosis de enmienda lo cual es consistente con la velocidad de disipación más alta de tebuconazol en los suelos enmendados debido a la formación de residuos enlazados que origina una disminución de su biodisponibilidad.

Durante el periodo de disipación de los fungicidas se consideró de interés determinar los cambios producidos en la actividad deshidrogenasa (DHA) de los suelos no enmendados y enmendados, no tratados y tratados con los fungicidas. La DHA se considera como un indicador muy útil de la actividad microbiana global del suelo y su evaluación es de interés para conocer el impacto de la enmienda, la aplicación de pesticidas y el tiempo de incubación sobre la actividad microbiana del suelo.

Los resultados obtenidos (Figura 4 de Anexos, página 205) muestran que la adición de SMS al suelo aumenta la DHA del suelo indicando el efecto de la enmienda para estimular la actividad microbiana del suelo aunque una disminución significativa de este efecto fue observada a lo largo del tiempo de incubación independientemente del suelo y tratamiento estudiado. Tampoco se observó ningún efecto significativo ($p > 0.1$) de los fungicidas aplicados sobre la DHA, a pesar de que al comienzo de la incubación los suelos tratados con tebuconazol presentaron una DHA mayor que los suelos control (sin fungicida). La actividad microbiana podría ser activada por una fuente extra de carbono aportada por la adición de pesticidas y enmiendas simultánea aunque algunos trabajos han puesto de manifiesto también una reducción de la actividad microbiana debido a efectos negativos de los fungicidas sobre las comunidades de hongos del suelo.

3.2.5. Estudio de la movilidad de pesticidas en suelos de viñedo de La Rioja no enmendados y enmendados con residuo postcultivo de champiñón

El objetivo de este trabajo fue estudiar la movilidad de fungicidas en columnas de suelos no enmendado y enmendado con SMS y la influencia en este proceso de diferentes factores como el flujo de lavado (saturado o saturado-no saturado) o el estado del fungicida en el suelo (no incubado o incubado). Considerando que el SMS podría ser usado como un potencial instrumento para inmovilizar pesticidas en suelos se planteó la necesidad de conocer cómo podría afectar al transporte posterior de los compuestos inmovilizados.

Se seleccionaron dos fungicidas con propiedades muy distintas: tebuconazol clasificado como no polar e inmóvil y cimoxanilo polar y móvil. El estudio de movilidad se llevó a cabo en columnas de suelo sin enmendar y enmendado con SMS al 5% y al 50%, la dosis más baja podría corresponder a la aplicación del SMS como enmienda orgánica en la agricultura y la dosis más elevada a la aplicación del SMS como barrera para evitar contaminaciones puntuales del suelo. Se aplicaron dos regímenes de lavado: saturado o flujo continuo de 12 volúmenes de poro (PV) de lavado y saturado-no saturado o flujo discontinuo de 12 PV de lavado. Los ensayos de movilidad se llevaron a cabo 1 día después de la aplicación del fungicida en las columnas (no incubadas) y después de 30 días de incubación o envejecimiento en las mismas (incubadas). Paralelamente se obtuvieron también curvas de lixiviación del ión trazador (Cl^-) en todos los suelos estudiados aplicando los dos flujos de lavado indicados con el fin de estimar si la movilidad de los fungicidas podría ser afectada con los flujos de lavado ensayados.

Las curvas de lixiviación del ion trazador (BTCs) se han incluido junto con las de los fungicidas en las Figuras 1 y 3 de Anexos (páginas 217 y 222). Todas son simétricas alcanzando el pico máximo a 1 PV y presentando una concentración de pico (concentración máxima del ion lixiviado durante el ensayo de movilidad) que se ve disminuida con la aplicación de la enmienda y bajo régimen saturado. La obtención de un pico máximo alrededor de 1 PV indica que no se produce retención del ion en suelos, además al final del ensayo se recuperó el 100% del Cl^- aplicado, indicando que la movilidad del ion trazador no se ve afectada por el tipo de flujo de lavado aplicado en la columna.

Las BTCs del tebuconazol obtenidas bajo las condiciones estudiadas se pueden observar en la Figura 1 de Anexos (página 217). En las columnas de suelo no enmendado el pico máximo se alcanzó en un rango de valores similares al ion trazador (1.14-1.41 PV), pero presentó menor concentración (4.92-3.52 %). Las curvas mostraron largas colas con hombros cuando se aplica el flujo saturado o presentan varios picos en el caso del flujo saturado-no saturado. La tendencia asimétrica de las curvas refleja una interacción dependiente del tiempo entre el tebuconazol y los componentes del suelo.

Las BTCs del fungicida en los suelos enmendados presentaron también picos de concentración máxima a un PV entre 1.06-1.35 (S+SMS5) y 1.08-2.15 (S+SMS50) (Tabla 4, página 218), con unas concentraciones máximas entre 2.25-3.95 % (S+SMS5) y 1.61-1.77% (S+SMS50). La tendencia que presentaron las curvas fue similar cuando el fungicida fue incubado en las columnas aunque la concentración del pico disminuyó. A pesar de que las constantes de adsorción fueron mayores en los suelos enmendados (Tabla 2), se observa una cinética de lixiviación más lenta del tebuconazol en el suelo no enmendado que puede sugerir una interacción más fuerte del tebuconazol con el suelo. Sin embargo, el lavado de un compuesto no retenido por el suelo en el primer PV no podría justificarse por el tebuconazol de acuerdo con los datos de la literatura para este compuesto por lo que podría corresponder a un metabolito del tebuconazol con una alta solubilidad en agua y un bajo coeficiente de adsorción, el cual podría estar presente como una impureza en el producto radioactivo empleado. Este compuesto mostraría un comportamiento similar al tebuconazol, siendo más adsorbido en el suelo enmendado, explicando así la disminución del pico máximo en relación con el suelo no enmendado.

De acuerdo con las constantes de adsorción de tebuconazol por el suelo no enmendado y el S+SMS previamente obtenidas, son las cantidades de tebuconazol lixiviado expresada como porcentaje de la cantidad de ^{14}C inicialmente añadida. Estas cantidades fueron mayores en el suelo no enmendado que en los suelos enmendados. Además las cantidades lixiviadas fueron más altas cuando se aplicó el flujo de lavado en régimen saturado. Por el contrario estas cantidades disminuyeron cuando el fungicida fue incubado en la columna de acuerdo con resultados previos obtenidos en el estudio del mecanismo de disipación de tebuconazol en los S+SMS y con el aumento de adsorción al aumentar el tiempo de contacto del fungicida en el suelo. Las cantidades

lixiviadas se corresponden con las cantidades retenidas en las columnas, siendo menores en los suelos sin enmendar (24.2-50.7%) que en los suelos enmendados variando entre 65.4-86.9% para el S+SMS5 y entre 87.6-94.9% para el S+SMS50 (Tabla 4 de Anexos página 218). La Figura 2 de Anexos (página 220) muestra la distribución del pesticida en los diferentes segmentos en los que se dividió la columna, observando que la retención del compuesto se produce mayoritariamente en el primer segmento de la columna.

El factor de retardo (R) se calculó con el fin evaluar los cambios en los picos máximos de las BTCs del fungicida lixiviado en todas las columnas estudiadas. Los valores calculados se muestran en la Tabla 2 (página 215), correspondiendo los valores más altos al S+SMS50 debido al aumento de la adsorción relacionada con el aumento del OC de este suelo y en línea con la correlación significativa encontrada entre adsorción y OC de los suelos enmendados.

Las BTCs para cimoxanilo incluidas en Figura 3 de Anexos, página 222, fueron en general asimétricas, pero se observaron diferentes tendencias en función de la dosis de SMS añadida y el flujo aplicado, indicando un comportamiento diferente del fungicida en función del sistema estudiado.

En las columnas de suelo no enmendado, las BTCs de cimoxanilo fueron simétricas y similares a las del ion trazador. Las concentraciones del pico máximo se obtuvieron para un PV próximo a 1 (entre 0.97 y 1.56) para los flujos saturado y saturado-no saturado. Sin embargo, las concentraciones máximas obtenidas variaron en función del periodo de incubación, representando cerca del 39% del fungicida aplicado a la columna en condiciones de no incubación y observándose un descenso en esta concentración cuando el fungicida fue incubado en la columna (12.9-23.1%). En todos los casos la lixiviación total del fungicida tuvo lugar para un volumen de lavado próximo a 3 PV. La forma que adoptan estas curvas es similar a la del ion trazador, indicando una rápida cinética de lixiviación debido a la débil interacción entre el fungicida y el suelo.

Las BTCs de cimoxanilo en los S+SMS presentaron menor simetría que en el suelo no enmendado. La presencia de colas al final de la curva indica un proceso de lixiviación más lento que en el suelo no enmendado. En este caso los picos de las BTCs

mostraron un retraso con respecto a las curvas de lixiviación del ion trazador (1.28-2.84 PV). Las concentraciones máximas de cimoxanilo lixiviado disminuyeron cuando el compuesto fue incubado, posiblemente debido al incremento de la adsorción por el S+SMS (Tabla 5 de Anexos, página 224).

Las cantidades totales de cimoxanilo lixiviado y retenido en la columna después del ensayo expresadas como porcentajes de la cantidad de ^{14}C -cimoxanilo aplicado se incluyen en la Tabla 5 de Anexos (página 224). La cantidad de cimoxanilo lavado fue próxima al 100% después de la aplicación de un volumen equivalente a 12 PV en flujo saturado, en los suelos enmendados y en los no enmendados, indicando que no se produce retención del compuesto en la columna. Sin embargo, la lixiviación disminuyó bajo flujo saturado-no saturado y además, esta disminución fue mayor cuando el compuesto fue incubado en las columnas. Esta disminución de las cantidades lixiviadas fue acompañada por un aumento en la mineralización del fungicida al aumentar el periodo de permanencia en la columna. Las cantidades mineralizadas fueron muy similares después de la aplicación de los dos flujos: 18% (S), 12% (S+SMS5) y 10% (S+SMS50). Solo se observó retención de cimoxanilo en las columnas de S+SMS cuando un flujo saturado-no saturado fue aplicado siendo la retención mayor en el primer segmento de la columna.

Al igual que en el caso del tebuconazol el factor de retardo (R) fue estimado para evaluar cambios en los picos máximos de las BTCs (Tabla 3 de Anexos, página 215). Los valores más altos para este parámetro se han obtenido para el S+SMS50 indicando la baja movilidad del cimoxanilo debido a la alta adsorción de este por el suelo con alto contenido en OM.

3.3. EFECTO DE LA APLICACIÓN SIMULTÁNEA DE PESTICIDAS Y SUSTRATO POSTCULTIVO DE CHAMPIÑÓN EN LAS COMUNIDADES MICROBIANAS DEL SUELO

3.3.1. Materiales

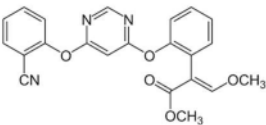
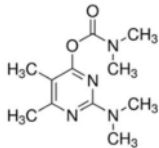
- **Suelos**

Se utilizó para este estudio el suelo de viñedo localizado en Alcanadre utilizado en los estudios anteriormente descritos e incluidos en el apartado 3.2. de esta memoria. La caracterización de este suelo ha sido descrita en el apartado 3.2.1 de la memoria.

- **Pesticidas**

Los pesticidas seleccionados fueron el insecticida pirimicarb y el fungicida azoxistrobin. Pirimicarb es un insecticida y fue uno de los pesticidas considerados para el estudio de adsorción de pesticidas por suelos enmendados con diferentes dosis de SMS y azoxistrobin es un fungicida y fue seleccionado para comparar los resultados obtenidos en suelos enmendados con SMS y en suelos enmendados con otros residuos orgánicos publicados en la literatura (Sopeña y Bending, 2013). Las características de ambos compuestos se incluyen en la Tabla 10. Azoxistrobin y pirimicarb son compuestos polares e inmóviles de acuerdo con la clasificación establecida por Delle Site (2001).

Tabla 10. Estructura química y propiedades fisicoquímicas de los pesticidas estudiados

| Nombre común | Tipo | Estructura | PM | Sol. Agua | Log | Koc (mL | DT ₅₀ | Índice |
|--|-----------------------------|--|------------------------|-----------------------|-----|-------------------|-----------------------|--------|
| Nombre IUPAC | pesticida | química | (g mol ⁻¹) | (mg L ⁻¹) | Kow | g ⁻¹) | (días ⁻¹) | GUS |
| Azoxistrobin | | | | | | | | |
| metil(<i>E</i>)-2-{2-[6-(2-cianofenoxi)pirimidin-4-yloxi]fenil}-3-metoxiacrilato | Fungicida (strobilurina) |  | 403.4 | 6.7 | 2.5 | 589 | 250 | 2.65 |
| Pirimicarb | | | | | | | | |
| 2-dimetilamino-5,6-dimetilpirimidin-4-yl dimetilcarbamato | Insecticida (carbamato) |  | 238.39 | 3100 | 1.7 | 388 | 86 | 2.73 |

Fuente PPDB, Pesticide Properties DataBase (2015). Sol. Agua, solubilidad en agua a 20 °C; Kow, coeficiente de partición octanol/agua a pH 7 y 20°C; Koc, coeficiente de adsorción normalizado al contenido de carbono orgánico, DT₅₀ tiempo de vida media de degradación aeróbica en suelo; Índice GUS, indicador de movilidad potencial.

3.3.2. Métodos experimentales

- **Preparación de suelos enmendados**

El suelo de Alcanadre se enmendó con el SMS a dos dosis: 2% y 5% (en peso seco) como se ha indicado en el apartado 3.2.2. de esta memoria. Las características de los suelos enmendados se incluyen en la Tabla 8. Los suelos fueron incubados a 20°C durante 24 horas antes de ser utilizados en los ensayos.

- **Cinéticas de disipación de pesticidas: Diseño del experimento y determinación de los pesticidas**

Los pesticidas estudiados se aplicaron a los suelos enmendados a la dosis agronómica (2 mg kg⁻¹) y a una dosis 12.5 veces mayor (25 mg kg⁻¹). La dosis más elevada se incluyó en el estudio con el fin de poder observar, en mayor medida, posibles alteraciones en la estructura de la comunidad microbiana. Para llevar a cabo el experimento se prepararon muestras individuales para evitar contaminaciones entre las muestras, de 50 g de suelo con una humedad correspondiente al 40% de la capacidad de campo. El contenido de humedad del suelo se mantuvo por adición de agua estéril cuando fue necesario. Las muestras se incubaron durante diferentes periodos de tiempo (0, 30 y 90 días) a 20°C. En cada uno de los tiempos se llevaron a cabo una extracción de los pesticidas con metanol; para ello 5 g de cada muestra incubada se agitaron durante 24 h con 10 mL de metanol. En el estudio se incubaron un total de 162 muestras resultado de los 3 tratamientos estudiados (suelo no enmendado, y enmendado con dosis de SMS del 2 y 5%, utilizando 3 dosis de aplicación de los pesticidas (0, 2 y 25 mg kg⁻¹), 2 pesticidas, 3 periodos de muestreo y 3 repeticiones).

Para determinar la persistencia de los pesticidas en los suelos no enmendados y enmendados se llevaron a cabo extracciones de los pesticidas con metanol. Muestras de 5g de los suelos incubados se agitaron con 10 mL de metanol durante 24 h y los pesticidas extraídos desde el suelo se determinaron mediante LC-MS/DAD. En la Tabla 11 se indican las condiciones experimentales. Los valores de vida media de los pesticidas (DT₅₀) se calcularon a partir de las ecuaciones cinéticas que mejor se ajustan a las curvas de disipación indicadas en el apartado 3.2.2.2.

Tabla 11. Condiciones cromatográficas establecidas para la detección de los pesticidas

| Condiciones | Azoxistrobin | Pirimicarb |
|----------------------------------|--|--|
| Fase móvil | 20:80 (H ₂ O+fórmico 0.1% : ACN) | 10:90 (H ₂ O+fórmico 0.1% : ACN) |
| Flujo (mL min ⁻¹) | 0.4 | 0.4 |
| Vol. inyec. (μl) | 20 | 20 |
| λ (nm) | 200 | 244 |
| m/z | 404.0 | 239.29 |
| t _R (min) | 6.3 | 6.3 |

- **Determinación de bioindicadores de comunidades microbianas de carácter general**

Se determinaron dos bioindicadores de carácter general, la actividad deshidrogenasa (DHA) y la respiración del suelo, para dar información sobre el impacto de la aplicación simultánea del SMS y los pesticidas en las comunidades microbianas del suelo.

La DHA es un indicador de la actividad total de las comunidades microbianas y se midió en muestras de suelo sin enmendar y enmendado, tratadas y sin tratar con los pesticidas estudiados. Para la determinación de la **DHA** se siguió el método de Tabatabai (Tabatabai, 1994) indicado anteriormente.

La **respiración** es un indicador del nivel de estrés que una perturbación puede provocar en los microorganismos del suelo. Un aumento de dicho coeficiente es indicador de la alteración del metabolismo de los microorganismos, los cuales se ven obligados a utilizar el carbono que asimilan para obtener energía mediante la respiración celular en lugar de para crear nuevas estructuras celulares (Marinozzi, 2013). La respiración fue determinada a través de la medida de absorción de O₂ por los

microorganismos presentes en muestras de 50 g de suelo incubadas a 25 °C durante 60 h usando el equipo OxiTop Control (WTW, Weilheim, Alemania) de acuerdo con la normativa DIN ISO 16072 (Figura 12).



Figura 12. Equipo de medida de la respiración del suelo

- **Determinación de la estructura de las comunidades microbianas del suelo:**
Técnicas de biología molecular

Las técnicas de **secuenciación de ADN** permiten la determinación de las poblaciones que se encuentran en un hábitat determinado. Se utilizó en este trabajo para evaluar el impacto de la aplicación simultánea de SMS y pesticidas sobre la comunidad microbiana del suelo. La técnica consiste en: 1) extraer el ADN presente en las muestras de suelo, 2) amplificar unas regiones del ADN mediante PCR que son específicas de cada especie, 3) construir las librerías de ADN con las secuencias amplificadas mediante una segunda PCR, 4) secuenciar las regiones amplificadas para 5) identificar que organismos están presentes y en qué cantidad relativa.

Para la **extracción de ADN** se tomaron 0,5 g de suelo tanto enmendados como sin enmendar mediante el uso del kit “FastDNATM SPIN kit for soil” (MPBio). La cuantificación del ADN se realizó mediante la medición con Qubit (Qubit ® BR DNA kit, Life Technologies Corporation) para normalizar las concentraciones extraídas a 5 ng/mL.

El ADN extraído se utilizó como molde para las PCR. Una PCR (reacción en cadena de la polimerasa) es una técnica ampliamente utilizada en biología molecular que permite la **amplificación** de regiones determinadas del genoma definidas mediante unos oligonucleótidos complementarios específicos. Para completar la reacción se

necesita la enzima polimerasa, el conector magnesio (normalmente incluido en el buffer de reacción), los oligonucleótidos específicos y el ADN molde del cual se va a realizar la amplificación. Para la primera PCR se utilizaron 1.25 µl de cada primer (Tabla 12), 12.5 µl de 2xQ5readmix, 7.5 µl de agua y 2.5 µl del ADN extraído y normalizado previamente de acuerdo al productor Q5® Hot Start High-Fidelity PCR Kit (New England Biolabs ® inc.). Los primers usados amplifican el gen 18S del ARN ribosómico, cuya secuencia específica permite identificar de que organismo proviene (Amaral-Zettler et al., 2009). El producto de PCR se volvió a cuantificar mediante Quibit y se procedió al protocolo de purificación previo a la secuenciación Illumina-MiSeq, realizando dos purificaciones con AMPure XP beads y el kit Qiagen. Después de la purificación se llevó a cabo una segunda PCR con primers específicos para Illumina-MiSeq, i5 e i7 suministrados por Illumina Nextera® Index Kit. Estos primers específicos incorporan en los extremos de la zona amplificada una secuencia determinada que permite identificar el origen de la muestra e hibridar con una secuencia complementaria en la placa de secuenciación. La concentración de ADN se normalizó a 10 nM y las muestras se enviaron a TGAC (The Genome Analysis Centre, Norwich, UK) para llevar a cabo la secuenciación Miseq v2 2 x 250 bp.

Tabla 12. Secuencia de los primers utilizados para la amplificación del 18S

| Primer | Secuencia |
|------------------|--|
| 18SEuk_1 391f | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTACACACCG CCCGTC |
| 18SEukBr | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGATCCTTCT GCAGGTTCACCTAC |

Para la secuenciación del ADN se utilizó la tecnología **illumina MiSeq**, que es un tipo de secuenciación por síntesis, presenta la ventaja de que permite la secuenciación paralela de multitud de muestras detectando la incorporación de bases nitrogenadas marcadas con moléculas fluorescentes a la hebra en crecimiento. En los últimos años esta tecnología se ha convertido en el método de referencia para estudiar comunidades microbianas.

El **análisis de los datos** procedentes de la secuenciación se realizó con el software QIIME (Caporaso et al. 2010), asignando cada unidad operacional taxonómica

(OTU) a una clasificación taxonómica. Las similitudes entre comunidades se analizaron mediante NMDS (non-metric multidimensional scaling approach) con el programa estadístico PRIMER 6.1.9 (PRIMER-E Ltd., Plymouth, UK). Con este mismo programa se realizó el análisis ANOSIM para identificar las diferencias en el perfil de las comunidades de los suelos enmendados o no enmendados, tratados con concentraciones diferentes de pesticida y durante diferentes tiempos de incubación (Clarke, 1993). Simultáneamente se utilizó el análisis SIMPER para especificar qué OTUs contribuían en la variación de las comunidades (Clarke and Warwick, 2001).

3.3.3. Estudio de los cambios en las comunidades eucariotas de un suelo no enmendado y enmendado con residuos postcultivo de champiñón en presencia de pesticidas.

La adición de enmiendas orgánicas al suelo puede aumentar o disminuir la disipación de pesticidas y puede modificar a su vez la fracción biodisponible del mismo en el suelo enmendado. La fracción biodisponible es un factor determinante en la acción del pesticida sobre las comunidades microbianas del suelo. Este trabajo se planteó debido a la necesidad de estudiar cómo pueden alterar tanto los pesticidas y las enmiendas la microflora endógena de suelos agrícolas. Para ello se llevó a cabo el estudio de la disipación de los pesticidas, pirimicarb y azoxistrobin, en un suelo agrícola no enmendado y enmendado con residuo postculivo de champiñón (SMS) a la dosis del 2 y 5%.

La disipación de ambos pesticidas siguió una cinética de primer orden. En todos los casos la disipación del azoxystrobin fue más lenta que la del pirimicarb (Tabla 1, de Anexos, página 233). En general para los dos pesticidas la disipación fue más rápida en el suelo enmendado y fue más lenta cuando los compuestos se aplicaron a dosis más elevadas. Estos resultados podrían indicar que la enmienda aumenta la actividad microbiana favoreciendo la degradación de estos dos pesticidas. En caso del azoxistrobin aplicado a dosis alta la disipación fue más lenta en los suelos enmendados lo que podría indicar una alta inmovilización del compuesto sin quedar expuesto a la degradación microbiana. Estudios en la literatura indican que una mayor interacción de azoxistrobin en el suelo enmendado podría producirse por una disminución del pH del suelo originado por la aplicación del SMS además del aumento del contenido en OC.

La DHA del suelo fue analizada como un indicador a gran escala del impacto que los pesticidas y la enmienda pueden causar sobre las comunidades microbianas del suelo (Figura 1 de Anexos, página 234). La DHA aumentó significativamente ($p < 0.001$) cuando se añadió la enmienda. Estos cambios se podrían explicar como ya se ha indicado también en experimentos anteriores debidos a la incorporación de una fuente de carbono extra procedente de la enmienda aplicada y produciendo al mismo tiempo cambios fisicoquímicos en las propiedades del suelo.

En el caso del pirimicarb, además se observa una disminución de la DHA en función del tiempo que se acentúa con la concentración del insecticida, indicando una baja resistencia de la comunidad microbiana a la aplicación de pirimicarb. Este comportamiento podría ser debido a la muerte o inhibición metabólica de una fracción microbiana sensible al pirimicarb a altas dosis. Este efecto no se observa en ninguna muestra cuando se aplica azoxystrobin a pesar de la persistencia mayor de este compuesto en el suelo. Los resultados de secuenciación obtenidos en este trabajo indicarían la posible evolución de las comunidades microbianas hacia tipos más tolerantes que no se ven afectadas por los pesticidas.

Los resultados obtenidos de los análisis de respiración de los suelos sin enmendar muestran el mismo comportamiento con ambos pesticidas (Figura 2 de Anexos, página 235). Además, si se compara el suelo sin enmienda con el enmendado, este último presenta valores más altos de respiración al final del periodo de incubación. El perfil de la respiración es el mismo para los dos pesticidas en los suelos enmendados. Inicialmente los valores más altos se alcanzan con las dosis más altas de pesticidas, pero esto se invierte a lo largo de la incubación alcanzando los suelos sin pesticida los valores más altos al final del experimento. En un primer momento los organismos resistentes al pesticida podrían utilizar este como una fuente de carbono o utilizar como nutrientes los organismos que no han sido resistentes al pesticida, equilibrándose a lo largo de la incubación.

Con el objetivo de analizar las variaciones en las comunidades de eucariotas del suelo se realizó la técnica Illumina Miseq al ADN extraído de los suelos estudiados. Esta permite observar la influencia de la presencia de pesticida, de la enmienda y del tiempo de cultivo en las comunidades del suelo. La técnica de secuenciación utilizada nos permite conocer la estructura del suelo, es decir la riqueza y composición de las

comunidades eucariotas del mismo. A su vez, mediante el análisis comparativo de los distintos suelos, se pueden conocer las diferencias entre las especies eucariotas encontrados en los mismos.

En los suelos tratados con azoxistrobin se detectaron un total de 5119 unidades taxonómicas operacionales (OTUs) de las cuales 943 se correspondieron con hongos.. En los suelos tratados con pirimicarb el número total de OTUs 6293 de los cuales 1185 se asignaron a hongos.

Los análisis comparativos de la riqueza de las unidades taxonómicas operacionales revelaron que la presencia de enmienda aumentaba significativamente la cantidad de eucariotas del suelo (Figura 4 de Anexos, página 238). No obstante no se vio efecto significativo en la riqueza de las unidades taxonómicas operacionales al tener en cuenta la tasa de la enmienda, el tiempo de incubación o la presencia de pesticidas.

El estudio de las diferencias de la composición de las unidades taxonómicas operacionales mostró de nuevo la influencia de la enmienda y también la importancia de la dosis de esta. Esto indica la incorporación de nuevas especies y/o la variación de las presentes. Además, se encontraron variaciones significativas al estudiar la influencia del tiempo de cultivo en las distintas dosis de enmienda, apoyando que el suelo es un ente dinámico que varía en el tiempo. Es interesante destacar que no se encontraron diferencias significativas que indiquen que la presencia de los pesticidas afecten a las comunidades eucariotas estudiadas, lo que probablemente se deba a que estas, al estar en suelos agrícolas acostumbradas a la presencia de estos pesticidas, se habrían seleccionado adaptándose a estos compuestos.

El estudio permitió también identificar los taxones más abundantes en las muestras (Figura 5 de Anexos, página 239), que fueron *Viridiplantae*, *Amoebozoa*, *Metazoa*, *Fungi* y *Heterolobosea*. Además se pudieron estudiar cuales son los microorganismos responsables de las variaciones encontradas comparando las distintas variables del experimento. De este modo se encontró un aumento en el tiempo de microartropodos y protistas y una disminución en residuos vegetales, pudiendo estos haber sido usadas a lo largo de la incubación como una fuente de nutrientes. Por otro lado, comparando suelos no enmendados y enmendados se observó un aumento en los

restos vegetales *Viridiplantae*, así como en *Agaricomycetes*, donde se encuentra el champiñón, principal componente de la enmienda.

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4. CONCLUSIONES

4. CONCLUSIONES

1. Se ha desarrollado un método multiresiduo para el análisis de residuos de más de 50 pesticidas y algunos de sus productos de degradación en aguas subterráneas y superficiales de un área de viñedos de la región de La Rioja basado en la extracción en fase sólida (SPE) y análisis por cromatografía de gases y/o líquidos con detector de espectrometría de masas. Las recuperaciones más altas se consiguieron cuando un volumen de agua de 500 mL fue concentrado usando cartuchos Oasis HLB y acetonitrilo-acetona como disolventes de elución. Los niveles de detección conseguidos para la mayor parte de los compuestos seleccionados permitieron su detección a los niveles establecidos por la legislación europea (0.1 µg/L).

2. La mayor parte de los 34 pesticidas estudiados en 25 muestras de aguas subterráneas analizadas mediante GC-MS o de los 58 pesticidas estudiados en 92 muestras de aguas superficiales y subterráneas analizadas mediante LC-MS, incluyendo algunos de sus productos de degradación, fueron detectados a concentraciones superiores al límite establecido por la comunidad europea en la mayoría de las muestras analizadas, lo que pone de manifiesto la presencia generalizada de los pesticidas en el área de estudio. Tanto el herbicida terbutilazina como su metabolito desetilterbutilazina (DET) y el fluometuron fueron detectados en el 96% de las muestras analizadas. Los fungicidas pirimetanil, ciprodinil, tebuconazol, kresoxim metil, metalaxil y sus metabolitos (CGA-62826 y CGA-92370) se detectaron en más del 50 % de las muestras.

3. La evaluación de 17 residuos de pesticidas y alguno de sus productos de degradación en 17 suelos de viñedos de la región de La Rioja se llevó a cabo mediante el desarrollo de un protocolo de extracción y determinación de pesticidas mediante GC-MS y LC-MS. La presencia del fungicida metalaxil, los herbicidas terbutilazina, su metabolito desetilterbutilazina, y fluometuron y los insecticidas pirimicarb y metoxifenocida fue especialmente frecuente en los suelos. Todos los pesticidas estudiados se encontraron en al menos una de las muestras analizadas con un 68% de muestras con detecciones positivas, aunque solamente en el 9% presentaron concentraciones por encima del límite de cuantificación.

4. Los resultados del análisis de residuos de pesticidas encontrados en aguas y suelos ponen de manifiesto la necesidad de implementar estrategias para prevenir y controlar contaminación difusa y puntual de las aguas y suelos por estos compuestos como consecuencia de su aplicación en áreas agrícolas.

5. Se ha utilizado el residuo postcultivo de champiñón (SMS) como una estrategia de bajo coste adecuada para prevenir la contaminación de suelos por pesticidas ya que facilita la inmovilización de estos compuestos en el suelo. La aplicación de SMS al suelo a diferentes porcentajes indicaron un aumento de 3 a 5 veces de las constantes de adsorción de pesticidas no polares (tebuconazol y triadimenol) y de 5 a 15 de las constantes de adsorción de pesticidas polares (cimoxanil y pirimicarb) cuando SMS se aplicó a dosis $\leq 10\%$ (similar a las dosis agronómica) y más que 20 veces para ambos tipos de pesticidas cuando se aplica a dosis $\geq 25\%$ (similar al uso de SMS como una barrera) debido al aumento del contenido en carbono orgánico suministrado por el biosorbente SMS.

6. Los resultados de adsorción estimados para suelos y SMS estaban de acuerdo con los datos experimentales de adsorción principalmente para pesticidas no polares y se podrían usar para predecir la adsorción de estos compuestos por suelos enmendados con SMS con el fin de aplicar la dosis de biosorbente más adecuada para prevenir contaminaciones difusas o puntuales evitando el lavado de estos compuestos a las aguas subterráneas.

7. El estudio del mecanismo de disipación y la movilidad de los pesticidas en el suelo enmendado con SMS completó la información sobre la evolución en el tiempo de los pesticidas inmovilizados. Se observó un aumento en la velocidad de disipación “aparente” de los fungicidas estudiados tebuconazol y cimoxanilo en el suelo enmendado con SMS a dosis baja (5%) y alta (50%) debido al aumento de adsorción de estos compuestos por el suelo enmendado con SMS en una forma no extraíble o enlazada. Estos residuos enlazados aumentaron para tebuconazol en todos los suelos con el tiempo de incubación del sistema suelo+SMS+fungicida, pero disminuyeron para el cimoxanilo debido a que este compuesto fue mineralizado desde el comienzo del periodo de incubación.

8. Los resultados obtenidos del lavado de los fungicidas en columnas de suelo enmendado con dos dosis de SMS bajo diferentes condiciones de flujo y estado de los compuestos indicaron una disminución de las cantidades lavadas de tebuconazol (2-3 veces) en el suelo enmendado con SMS a dosis baja (5%) y alta (50%) cuando se aplicó un volumen de agua correspondiente a 12 VP con un flujo saturado o saturado-no saturado. La disminución fue mayor cuando el compuesto fue previamente incubado en la columna de suelo durante 30 días. El lavado de cimoxanilo fue más rápido que el de tebuconazol aunque el lavado total del compuesto encontrado en el suelo no enmendado disminuyó también hasta 2.6 veces en el suelo+SMS50 cuando se aplicó un flujo de lavado saturado-no saturado aunque se detectó mineralización del compuesto. El SMS disminuyó el lavado de ambos fungicidas en diferente medida, impidiendo el lavado de los residuos enlazados o no extraíbles de tebuconazol y disminuyendo la mineralización de cimoxanilo.

9. La utilización de técnicas de biología molecular para el estudio del efecto de SMS y pesticidas sobre las comunidades microbianas del suelo puso de manifiesto una clara influencia de la enmienda sobre las comunidades endógenas del suelo. No se encontraron diferencias significativas que indiquen que la presencia de los pesticidas afecten a las comunidades eucariotas estudiadas, posiblemente debido a la capacidad adaptativa de los microorganismos del suelo, y especialmente de los suelos agrícolas.

10. En consecuencia, diferentes dosis de SMS pueden aplicarse con el fin de diseñar estrategias para prevenir la contaminación difusa o puntual de los suelos y aguas. La aplicación de una dosis adecuada debería determinarse en función del tipo de suelo y especialmente considerando las características del pesticida ya que la aplicación de SMS puede contribuir a la disminución de la disipación y lixiviación de compuestos no polares al aumentar la cantidad adsorbida en forma de residuos no extraíbles o a la disminución de la mineralización en el caso de compuestos polares más biodisponibles en el suelo.

5. ANEXOS: TRABAJOS PUBLICADOS

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Pesticides and degradation products in groundwaters from a vineyard region: optimization of a multi-residue method based on SPE and GC-MS.

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Abstract

A reliable multi-residue method based on solid-phase extraction was developed using gas chromatography–mass spectrometry to determine and quantify thirty-four pesticides, including herbicides, fungicides, insecticides and some of their degradation products, in groundwater in a vineyard region of La Rioja (Northern Spain). Different parameters were optimized and good recoveries (65–108 % range) and precisions (12–19 % range) were achieved with spiked water samples for a concentration of 0.1 µg L⁻¹. The experimental results showed an excellent linearity ($r^2 > 0.99$) over the 0.1–1.5 µg L⁻¹ range. The detection limits of the proposed method were 1–37 ng L⁻¹ for most of the compounds studied. The methodology has been successfully applied to the analysis of groundwater samples from vineyard areas in La Rioja and the presence of pesticides, especially fungicides and herbicides, at several concentration levels was revealed. Terbutylazine, its metabolite desethyl-terbutylazine, and fluometuron were the pesticides most frequently detected in higher concentrations. Overall, and taking into consideration the European Union maximum residue limit of pesticides in groundwater, sixteen of the thirty-four compounds included in this study were detected in concentrations over that limit in at least one of the samples analyzed.

Keywords: Degradation products, groundwater, multiresidue analysis, pesticide pollution, vineyard region

1 INTRODUCTION

In recent decades, concern about the contamination of water sources has increased due to the rising number of pesticides detected. Pesticides are widely present in the environment, including water and soils, and in potential foodstuffs, as a result mainly of their application in modern agriculture. Furthermore, pesticides in the environment can be transformed into a large number of degradation products, commonly defined as transformation products (TPs), which can sometimes be as toxic and as persistent as the original pesticide [1].

The list of widely used pesticides is very long, but only some of them are regulated by the EU. Decision No. 2455/2001/EC [2] has established a list of 33 priority substances in the field of water protection, the third of which are pesticides. Several directives have set threshold values in order to control pesticide pollution in environmental waters. Council Directive 98/83/EC [3] has set the limits for

pesticides in water intended for human consumption at $0.1 \mu\text{g L}^{-1}$ for individual pesticides and $0.5 \mu\text{g L}^{-1}$ for the sum of all pesticides. More recently, Directive 2006/118/EC [4], which refers to groundwater pollution and deterioration, has set the same limits, albeit including active substances and their relevant metabolites, and degradation and reaction products.

Traditionally, conventional multi-residue analytical methods based on gas chromatography have been developed for the analysis of pesticides in water, although different detectors were necessary for good sensitivity depending on the pesticide family. For example, imidazole and pyrethroid compounds were detected with an electron capture detector (GC-ECD) [5, 6], while organophosphorus compounds were detected using a nitrogen-phosphorous detector (GC-NPD) [7,8]. However, the mass spectrometer is now considered the most suitable detection system for the multi-residue analysis of pesticides belonging to different chemical groups.

Analytical methods based on gas chromatography (GC) [9–11] or liquid chromatography (LC) [12–15], followed by mass spectrometry (MS) or tandem mass spectrometry, have been widely applied for determining semivolatile and volatile organic pollutants with satisfactory sensitivity and selectivity. They are the most commonly used techniques for the quantification of organic pesticides, although the quantification of these compounds in the environmental samples at the levels established by legislation (lower than the detection limits of the GC-MS method) requires a first step of extraction and preconcentration. A further difficulty is that the collection and sample preparation step takes up to two-thirds of the time required to perform the complete analysis [16].

Multi-residue analytical methods developed for the analysis of pesticides in water frequently use liquid–liquid extraction (LLE) [17] and solid-phase extraction (SPE) [18–21] as pre-treatment methods. Other more recently developed extraction techniques are solid-phase microextraction (SPME) [22, 23], stir bar extraction (SBE) [24] and single-drop microextraction (SDME) [25]. Among these pre-treatment methods, SPE is being increasingly used in water analysis because of its stability and variety of sorbents. SPE methods are rapid, and allow good recoveries and low detection limits of pollutants. In this sense, the optimization of the preconcentration method is an important process for recording the highest enrichment efficiency and the best recoveries. SPE sorbents were used for the clean-up/preconcentration of trace pesticides [26]. Furthermore, new kinds of SPE sorbents have recently appeared, such as molecularly imprinted polymers [27] and carbon-based nanomaterials [28].

The amount and kinds of pesticides in the water in a particular area depend largely on the intensity and type of crops being cultivated. However, the transport of pesticides beyond the area of application leads to the presence and accumulation of these compounds in many parts of the hydrosphere. Pesticide residues have been reported in water in different parts of Spain [21, 29–31], as well as in France [32], Italy [7] and Hungary [33], among others. The vineyard region of La Rioja is located in northern Spain, on both sides of the River Ebro. Every year, more than 250 million litres of wine are produced in this region and huge amounts of pesticides are used to treat the vines. La Rioja was the fifth Spanish region with the highest investment per hectare in crop protection products, with a consumption of 13.79 kg ha^{-1} in 2008 [34].

The objectives of this research were to develop and optimize a reliable multi-residue method based on SPE for determining and quantifying pesticides, along with its application for evaluating the

possible pollution of groundwater in La Rioja affected by vineyard and agricultural land with a high use of pesticides. To conduct the study, a set of thirty-four compounds, including pesticides belonging to different chemical classes and some of their main degradation products, were selected according to data provided by public bodies, plant protection product dealers and local farmers concerning the products used in the region in recent years (four of these thirty-four compounds are included in the priority list). A special effort has been made during optimization to strike a balance between efficient chromatographic separation and a short runtime, while still maintaining satisfactory sensitivity.

2 EXPERIMENTAL

2.1 Chemicals and reagents

The organic pesticides and their degradation products were purchased from Riedel de Haën (Seelze-Hannover, Germany) and Dr. Ehrenstorfer (Augsburg, Germany), and were used without further purification (minimum purity higher than 98 %). The compounds studied (including fungicides, herbicides and insecticides belonging to several chemical classes) are listed in Table 1. Stock solutions of all the pesticides (nominal concentration of 1000 or 500 $\mu\text{g mL}^{-1}$) were prepared by dissolving reference standards in methanol. Another stock solution containing all the compounds was prepared by volume dilution of suitable aliquots with methanol. These solutions were stored at 4 °C in the dark and were used for the preparation of dilute working standard solutions.

Acetone, acetonitrile, hexane and methanol in residue analysis grades were purchased from Fisher Scientific (Loughborough, UK). Ultrapure water (UHQ) was obtained from a Milli-Q water system (Millipore, Milford, MA, USA).

2.2 Apparatus and chromatographic conditions

GC/MS analysis was performed with an Agilent 7890 gas chromatograph coupled to an Agilent 5975 MSD mass spectrometer (Agilent Technologies, Wilmington, DE, USA). Chromatographic separation was performed on a 30 m \times 0.25 mm I.D., 0.25 μm film thickness DB-17MS capillary column (J&W, Avondale, USA). The optimum chromatographic conditions were as follows: the mass spectrometer was in the electron impact ionization mode, with an ionizing energy of 70 eV and an electron multiplier voltage set at 1700 V and a dwell time of 10 msec. Source and transfer line temperatures were adjusted to 230 °C and 280 °C, respectively. Helium was used as the carrier gas at a flow rate of 1.5 mL min^{-1} . The injector was operated in splitless mode, injecting 1 μL at 220 °C. The oven was programmed as follows: from 100 to 200 °C at 20 °C min^{-1} (holding time 1 min), to 210 °C at 1 °C min^{-1} , to 230 °C at 5 °C min^{-1} and to 315 °C at 50 °C min^{-1} (2.3 min), giving a total chromatographic time of 24 min. A solvent delay of 3 min was used to prevent damage to the filament in the ion source.

Acquisition was performed in the selected ion monitoring (SIM) mode according to the parameters shown in Table 1. Three product ions were selected among the more abundant, and one was chosen for quantification. The selected ions were checked with the retention time and required to abide by relative intensity of the qualifier ions in the ± 10 % range. Quantification was performed injecting each sample twice.

Table 1. List of compounds studied and GC/MS parameters used.

| Compound | SIM window | RT (min) | Monitored ions (Abundance) | |
|-----------------|------------|----------|----------------------------|-----------------------|
| | | | Target (m/z) | Qualifier Ions (m/z) |
| Fluometuron | 1 | 4.5 | 174 | 219 (794) / 187 (536) |
| CGA 92370 | 1 | 7.3 | 148 | 120 (572) / 91 (322) |
| Metobromuron | 1 | 7.8 | 229 | 231 (995) / 199 (497) |
| DET | 2 | 8.3 | 186 | 188 (319) / 83 (287) |
| DEA | 2 | 8.4 | 172 | 174 (321) / 187 (315) |
| DIA | 2 | 8.6 | 173 | 158 (870) / 145 (751) |
| Propazine | 2 | 8.8 | 214 | 229 (659) / 172 (622) |
| Diazinon | 2 | 8.8 | 179 | 137 (984) / 152 (704) |
| Atrazine | 2 | 9.2 | 200 | 215 (591) / 58 (389) |
| Terbuthylazine | 2 | 9.3 | 214 | 43 (574) / 173 (512) |
| Simazine | 2 | 9.5 | 201 | 44 (795) / 186 (624) |
| Pyrimethanil | 3 | 10.0 | 198 | 199 |
| Dimethoate | 3 | 10.4 | 87 | 93 (535) / 125 (454) |
| Acetochlor | 3 | 10.9 | 59 | 146 (839) / 162 (696) |
| Alachlor | 3 | 11.4 | 45 | 160 (378) / 188 (304) |
| Pirimicarb | 3 | 11.5 | 166 | 72 (877) / 238 (237) |
| Metalaxyl | 4 | 12.9 | 45 | 206 (326) / 132 (299) |
| Metolachlor | 4 | 13.1 | 162 | 238 (469) / 45 (197) |
| Terbutryn | 4 | 13.2 | 226 | 185 (838) / 170 (629) |
| Metribuzin | 4 | 13.3 | 198 | 57 (216) / 199 (190) |
| Chlorpyrifos | 4 | 13.6 | 197 | 199 (974) / 314 (865) |
| Ethofumesate | 4 | 14.0 | 207 | 161 (752) / 137 (376) |
| Penconazole | 5 | 16.3 | 248 | 159 (851) / 161 (577) |
| Triadimenol | 5 | 16.4 | 112 | 128 (906) / 168 (745) |
| Cyprodinil | 5 | 16.5 | 224 | 225 (616) / 210 (100) |
| Flutriafol | 5 | 20.6 | 123 | 164 (552) / 219 (255) |
| Myclobutanil | 5 | 21.4 | 179 | 82 (451) / 150 (449) |
| Kresoxim-methyl | 5 | 22.2 | 116 | 131 (533) / 206 (516) |
| Benalaxyl | 6 | 24.3 | 148 | 91 (574) / 206 (288) |
| Tebuconazole | 6 | 24.6 | 125 | 250 (847) / 70 (492) |
| Diclofop-methyl | 6 | 24.9 | 253 | 340 (800) / 255 (690) |
| Metamitron | 6 | 25.2 | 104 | 202 (864) / 174 (456) |
| Nuarimol | 6 | 25.3 | 107 | 139 (765) / 235 (744) |
| Lenacil | 6 | 26.0 | 153 | 53 (90) / 154 (86) |

2.3 SPE procedure optimization

The analytes from spiked water samples were preconcentrated by solid-phase extraction on an extraction manifold (Waters, Milford, MA, USA). To optimize sample pre-treatment, the following parameters were optimized: sorbent type, elution solvent and sample volume. Four different sorbents were compared for extracting the selected compounds: Oasis HLB cartridges (60 mg, Waters, Milford, MA, USA) and LiChrolut EN cartridges (200 mg, Merck, Germany) of polymeric sorbents, silica-based bonded C18 cartridges (Sep-Pak Plus 900 mg, Waters, USA) and graphitized carbon cartridges (SampliQ 500 mg, Agilent, USA). Each cartridge was conditioned with 5 mL of methanol and 10 mL of UHQ water. A 50 mL sample of UHQ water spiked with all the analytes at a concentration of $4 \mu\text{g L}^{-1}$ was passed through the cartridges at a flow rate of 7 mL min^{-1} by means of a peristaltic pump. Once the retention step had been completed, the cartridges were dried in an air current under a vacuum of $\mu 20 \text{ mm Hg}$ for 5 min. The components retained were eluted with 5 mL of methanol. The organic phase obtained was evaporated under a nitrogen stream at 45°C using an evaporator EVA-EC2-L (VLM GmbH, Bielefeld, Germany) until completely dry, and the dry residues obtained from the SPE assays were re-dissolved in 0.5 mL of methanol. The final extracts were filtered through $0.45 \mu\text{m}$ GHP Acrodisc filters (Waters Corporation) into GC vials and analysed.

The influence of the elution solvent was studied after selecting the SPE sorbent. Four different elution solvents were passed through the cartridges: methanol, acetonitrile, acetone and hexane. Extracts were obtained in the same way as for the previous experiment, although in this case the cartridge was conditioned with the corresponding solvent and UHQ water. The sample volume was then optimized once the type of cartridge and elution solvents had been selected. Different volumes of UHQ water (100, 250, 500 and 1000 mL) were spiked with the same amount of each pesticide ($0.2 \mu\text{g}$). Extracts were prepared in the same way as for the previous experiment.

2.4 Method performance and validation

To evaluate the influence of the matrix on the analysis, 500 mL of UHQ and groundwater (previously analyzed to check for the absence of any of the compounds studied) were spiked with all the compounds at a concentration of $0.2 \mu\text{g L}^{-1}$.

The linearity of the calibration curves was evaluated at a concentration range between 0.1 and $1.5 \mu\text{g L}^{-1}$ using eight calibration standards prepared in uncontaminated groundwater (matrix-matched calibration standards). Calculations were made using peak areas. The accuracy (average recovery) and precision (reproducibility and repeatability) of the method were assessed using pesticide-free groundwater spiked with all the pesticides at two spiking concentrations (0.1 and $0.5 \mu\text{g L}^{-1}$). Recoveries were determined for six replicates. The limits of detection (LODs) and quantification (LOQs) were estimated as the analyte concentration with a signal-to-noise-ratio of 3 and 10, respectively.

2.5 Analysis of real groundwater

Overall, 25 samples were taken in March 2010 from the vineyard region of La Rioja (Northern Spain). They were collected in 2 L amber glass bottles and immediately put on ice. Within four days, the samples were filtered through nitrocellulose filters with $0.45 \mu\text{m}$ pore size membranes (Millipore), being

kept refrigerated at 4 °C in the dark prior to extraction. Preconcentration of the analytes was performed by passing 500 mL of water through Oasis HLB cartridges. Two replicates of each sample were carried out.

3 RESULTS AND DISCUSSION

3.1 GC-MS optimization

Preliminary experiments for optimizing chromatographic conditions were performed using a methanol standard solution of 1 µg mL⁻¹, with the GC–MS operating in full scan. GC parameters that could affect peak resolution and analysis time, such as oven temperature programme and flow of the carrier gas, were evaluated and optimized. Although carrier gas flow changes did not have an important impact on the results, some differences were observed, especially in peak shapes. The best results were obtained with a flow of 1.5 mL min⁻¹, so this value was used for further experiments. Different temperature ramp rates with different initial and final temperatures were tested for rapid separation with adequate resolution; the best results were obtained with the conditions described in the experimental part (Section 2.2). Another parameter studied was the injection mode, with no significant differences being observed when injection was carried out in split mode with small ratios, or splitless and pulsed-splitless (split mode with high ratios produce smaller signals). Hence, splitless mode was selected for sample injection.

Once the GC–MS conditions had been optimized, the target and reference ions for each compound were selected. This step was carried out by comparing the spectrum obtained for each compound with the NIST library spectrum. Generally, the most abundant and/or characteristic ions were selected for identifying and quantifying the analytes. When the pesticide was not included in the library, as in the case of pyrimethanil, characteristic ions were selected from the injection of the standard. Compounds were sorted into groups (time window) to avoid degrading peak shape if the number of ions registered is too wide. The ions selected for each compound and time windows are shown in Table 1.

3.2 Optimization of SPE procedure

With a view to obtaining a more sensitive method for quantifying the pesticides and their metabolites in groundwater, different parameters that may affect SPE efficiency, such as the kind of sorbent, elution solvents and sample volume, were optimized.

To evaluate the retention capacity of the sorbents studied, a 50 mL sample of UHQ water spiked with all the analytes at a concentration of 4 µg L⁻¹ was passed through the sorbents. Figure 1 shows the recovery values obtained with the four cartridges for some of the compounds studied, which are representative of the behaviour of all the compounds in the different cartridges.

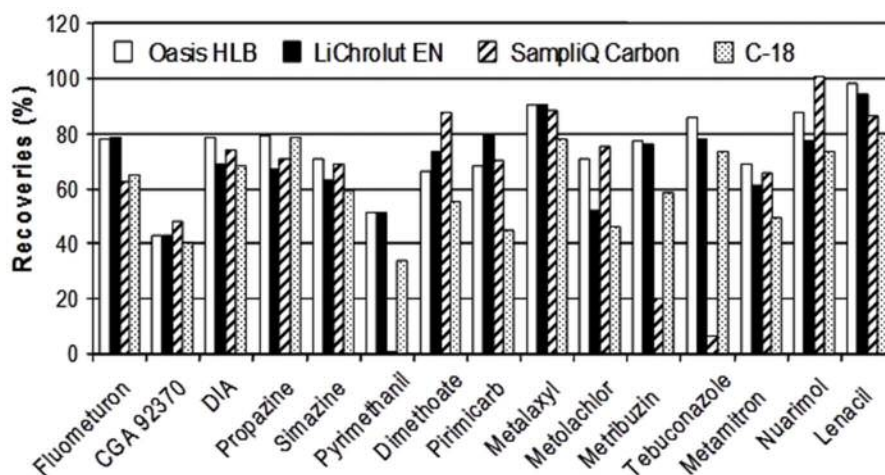


Figure 1. Effect of the SPE sorbent on recovery (R). Sample: 50 mL of ultrapure water spiked with $4 \mu\text{g L}^{-1}$ of each pesticide; cartridges were eluted with methanol.

The recoveries obtained with the Oasis HLB and LiChrolut EN sorbents were, in general, very similar (42-97 % range), although slightly higher recoveries were obtained for some compounds, such as triazines, metolachlor or nuarimol, when the Oasis HLB sorbent was used. When a carbon-based sorbent was used, the recoveries for most of the compounds were also quite similar to those obtained with Oasis or LiChrolut. However, recoveries were very low for several compounds, such as pyrimethanil, tebuconazole, metribuzin, diazinon and chlorpyrifos, which could be not retained or strongly retained in this kind of sorbent and not eluted. The recoveries obtained for all the compounds were significantly lower with C-18 sorbents (33.5-79.6 % range) than the values obtained with the polymeric sorbents.

According to the results obtained, the Oasis HLB and LiChrolut EN cartridges were selected to optimize analyte elution (Oasis HLB cartridges were used to analyze all the samples and LiChrolut EN cartridges to confirm the values of concentration found and to validate the method). Water samples (50 mL) spiked at a level of $4 \mu\text{g L}^{-1}$ for each compound were passed through the cartridges, and four different elution solvents were tested: methanol, acetonitrile, acetone and hexane. The results indicated that the recoveries with hexane for all the compounds were very low ($< 50\%$). Different behaviours were observed with the other solvents as a function of compound polarity. For more polar compounds, the best results were obtained with acetonitrile for Oasis and with methanol for LiChrolut, with these recoveries being 7-12 % higher than for the other solvents. For more hydrophobic compounds, the best results were obtained when acetone was used to elute both cartridges, with recoveries that were 5-16 % higher. It was therefore considered that elution with two solvents (4 mL of acetone + 4 mL of acetonitrile or methanol) could optimize the recovery of residues of different pesticides after water elution through Oasis or LiChrolut cartridges, respectively. Under these conditions, the recoveries were always higher than 65 % for all the compounds.

To optimize the enrichment factor and check for the absence of “breakthrough phenomena”, different volumes of UHQ water (100, 250, 500 and 1000 mL) were spiked with the same amount of each pesticide ($0.2 \mu\text{g}$) and passed through Oasis HLB cartridges. The results indicated that no remarkable differences were found in the recoveries obtained in the range of volumes studied. Only when the

preconcentrated volume was 1000 mL there was a significant decrease in recoveries obtained for all the analytes, being this decrease bigger for certain analytes, such as diazinon and metamitron. For this reason, and as a compromise between the enhancement of the enrichment factor and the experimental time and/or applicability for routine analysis, 500 mL was adopted as the sample volume for the extraction of pesticide residues.

3.3 Method performance and validation

To evaluate the influence of the matrix on the analysis, a sample of uncontaminated groundwater, previously analysed to check for the absence of the pesticides studied, was used to validate the optimized method. Following the preconcentration of both this sample and the UHQ water sample, and the elution of pesticides according to previously optimized conditions, the signals obtained for pesticides from both water samples were compared. More than 85 % of the compounds studied recorded very low signal suppression or enhancement (-15 % to +15 %). This variation in the signal ranged between 15-20 % solely for CGA-92370, desethyl-terbuthylazine (DET), myclobutanil, metribuzin and acetochlor. Despite this small matrix effect, matrix-matched standards were used for a more precise quantification of the concentration of these compounds in real groundwater.

Matrix-matched standards were prepared with 500 mL of environmental water and then spiked with all the analytes in the 0.1–1.5 $\mu\text{g L}^{-1}$ concentration range. Good linear relationships were obtained with regression coefficients (r^2) higher than 0.99 for all the compounds in the range studied. Only myclobutanil had a regression coefficient of 0.989 (Table 2).

The method was validated by evaluating the limits of detection (LODs) and quantification (LOQs), as well as the accuracy and precision at 0.1 and 0.5 $\mu\text{g L}^{-1}$ levels of pesticide concentration in uncontaminated environmental water. The LODs ranged between 1 and 37 ng L^{-1} for pyrimethanil and dimethoate, respectively, and the LOQs ranged between 5 and 87 ng L^{-1} for metalaxyl and dimethoate, respectively. Only chlorpyrifos and diclofop-methyl had an LOD and LOQ higher than the limit established by the EU for drinking water (Table 2). The accuracy and precision of the proposed method were determined by means of recovery experiments and the relative standard deviations (RSD) of the signals corresponding to six samples at two different concentration levels (0.1 and 0.5 $\mu\text{g L}^{-1}$) for each analyte. The recovery values were between 65-108 %, with RSD in the 12-19 % range for the lower concentration level, and between 61-107 %, with RSD in the 8-17 % range for the higher level. Recovery values were similar to those obtained in the optimization of the SPE when preconcentration was carried out in UHQ water, which confirms the absence of the matrix effect or its correction using matrix-matched standards.

Table 2. Analytical characteristics of the GC-MS method after SPE with Oasis HLB cartridges.

| Compound | Recovery (%) | RSD ^a (%) | Recovery (%) | RSD ^a (%) | r^2 (0.1-1.5 ^b µg L ⁻¹) | LOD ^c µg L ⁻¹) | LOQ ^d µg L ⁻¹) |
|-----------------|------------------------------|----------------------|------------------------------|----------------------|--|--|--|
| | 0.1 µg L ⁻¹ (n=6) | | 0.5 µg L ⁻¹ (n=6) | | | | |
| Fluometuron | 94 | 12 | 89 | 8 | 0.997 | 0.018 | 0.058 |
| CGA 92370 | 78 | 17 | 86 | 12 | 0.992 | 0.031 | 0.078 |
| Metobromuron | 85 | 15 | 82 | 11 | 0.991 | 0.026 | 0.059 |
| DET | 99 | 19 | 94 | 17 | 0.991 | 0.008 | 0.025 |
| DEA | 85 | 14 | 87 | 11 | 0.997 | 0.018 | 0.053 |
| DIA | 81 | 16 | 78 | 12 | 0.990 | 0.011 | 0.038 |
| Propazine | 79 | 15 | 84 | 15 | 0.996 | 0.012 | 0.037 |
| Diazinon | 82 | 16 | 80 | 10 | 0.991 | 0.022 | 0.056 |
| Atrazine | 81 | 14 | 84 | 16 | 0.997 | 0.016 | 0.048 |
| Terbuthylazine | 81 | 13 | 79 | 13 | 0.994 | 0.004 | 0.011 |
| Simazine | 79 | 16 | 81 | 9 | 0.992 | 0.013 | 0.034 |
| Pyrimethanil | 65 | 14 | 67 | 8 | 0.997 | 0.001 | 0.006 |
| Dimethoate | 83 | 17 | 80 | 14 | 0.990 | 0.037 | 0.087 |
| Acetochlor | 82 | 15 | 76 | 14 | 0.993 | 0.014 | 0.033 |
| Alachlor | 108 | 12 | 107 | 10 | 0.998 | 0.020 | 0.058 |
| Pirimicarb | 75 | 15 | 77 | 10 | 0.996 | 0.014 | 0.039 |
| Metalaxyl | 108 | 12 | 104 | 9 | 0.996 | 0.002 | 0.005 |
| Metolachlor | 81 | 15 | 78 | 11 | 0.998 | 0.008 | 0.023 |
| Terbutryn | 68 | 17 | 78 | 11 | 0.996 | 0.026 | 0.073 |
| Metribuzin | 81 | 18 | 89 | 12 | 0.998 | 0.021 | 0.064 |
| Chlorpyrifos | - | - | 83 | 14 | 0.992 | 0.108 | 0.219 |
| Ethofumesate | 78 | 12 | 76 | 11 | 0.996 | 0.010 | 0.028 |
| Penconazole | 83 | 14 | 85 | 11 | 0.993 | 0.005 | 0.021 |
| Triadimenol | 77 | 15 | 85 | 12 | 0.996 | 0.011 | 0.036 |
| Cyprodinil | 94 | 16 | 103 | 8 | 0.997 | 0.005 | 0.013 |
| Flutriafol | 80 | 17 | 78 | 10 | 0.999 | 0.003 | 0.013 |
| Myclobutanil | 83 | 15 | 87 | 9 | 0.989 | 0.016 | 0.041 |
| Kresoxim-methyl | 91 | 14 | 97 | 10 | 0.994 | 0.013 | 0.041 |
| Benalaxyl | 69 | 12 | 66 | 11 | 0.997 | 0.006 | 0.022 |
| Tebuconazole | 84 | 17 | 88 | 12 | 0.997 | 0.012 | 0.038 |
| Diclofop-methyl | - | - | 61 | 13 | 0.994 | 0.124 | 0.284 |
| Metamitron | 75 | 13 | 77 | 10 | 0.992 | 0.019 | 0.054 |
| Nuarimol | 83 | 14 | 91 | 8 | 0.995 | 0.013 | 0.030 |
| Lenacil | 97 | 14 | 102 | 12 | 0.999 | 0.017 | 0.043 |

^a RSD Relative standard deviation (n=6). ^b Lineal range. ^c LOD Detection limit for a signal-to-noise ratio of 3. ^d LOQ Quantification limit for a signal-to-noise ratio of 10

3.4 Application of the multi-residue method to real samples

The method developed was applied to the analysis of 25 groundwater samples taken from the vineyard region of La Rioja. Table 3 summarizes the results obtained (precision of the duplicate analysis was always lower than the precision measured for the validation of the method). All the samples analyzed contained two or more pesticide residues. Most of the compounds studied were detected in each one of the samples studied, and only metamitron, diclofop-methyl, metobromuron, alachlor, dimethoate, chlorpyrifos and the metabolite desethyl atrazine (DEA) were not detected in any of the samples. Figure 2 shows a chromatogram of one of the more contaminated samples, as indicated by the presence of several pesticides found in concentrations over the limit established by EU legislation.

The triazine herbicide terbuthylazine and its metabolite DET were detected in 96 % of the samples analyzed, even in concentrations ten times higher than the EU limit. However, other dealkylated metabolites of triazines were found only in fewer than 20 % of the samples (desisopropyl atrazine-DIA) or were not detected in any sample (DEA). The case of DIA is particularly interesting because it was found only in two groundwater samples in concentrations above $0.1 \mu\text{g L}^{-1}$, although it is also a degradation product of terbuthylazine, like DET. This result is consistent with the fact DET is the most abundant terbuthylazine degradation product, and DIA plays only a minor role among the degradation products of this herbicide [35]. The presence of triazines, such as atrazine and simazine, in groundwater confirmed it is still contaminated, although the use of this herbicide was banned in the EU in 2004. Other herbicides that were also detected in more than 50 % of the samples were metolachlor, ethofumesate and fluometuron.

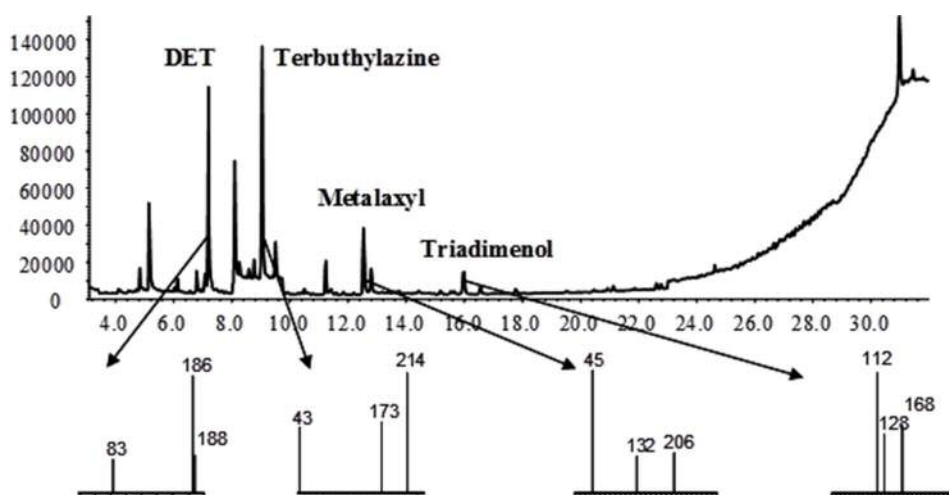


Figure 2. Chromatogram obtained after solid-phase extraction of a groundwater sample. Sorbent: Oasis HLB. Elution solvent: 4 mL of acetone + 4 mL of acetonitrile.

With respect to fungicides, we detected the presence of all the compounds studied in at least one of the sampled points. Several fungicides (pyrimethanil, cyprodinil, metalaxyl, tebuconazole and kresoxim-methyl) were found in more than 50 % of the samples analyzed. The metabolite of metalaxyl, CGA-92370, was found in 20 % of the samples studied, recording a concentration level higher than $1 \mu\text{g}$

L⁻¹ in one of the samples. Metalaxyl, its metabolite CGA-92370 and triadimenol were found in concentrations that were ten times higher than the EU limit.

The insecticides included in the study were found in a much smaller number of samples. Pirimicarb was found in 40 % of the samples, but in concentrations lower than 0.1 µg L⁻¹. Diazinon was also found in two of the samples, reaching a value of 0.135 µg L⁻¹ in one of them. This is consistent with the fact that these kinds of pesticides are the ones less used in the area studied.

To verify the results obtained, some of the samples were simultaneously analysed using the Oasis HLB and LiChrolut EN sorbents, following the same optimized procedure. A comparison of results (Table 4) showed that the concentrations of pesticides found using the Oasis HLB sorbent were similar to those obtained when using the LiChrolut EN cartridge. This confirms the reliability of the procedure proposed in this work.

Table 3. Percentage of groundwater samples with concentrations below, above, or ten times higher than the legislated maximum level for human consumption (0.1 µg L⁻¹), percentage of positive samples and concentration range of pesticides in 25 groundwater samples from the La Rioja region

| Compound | Percentage of samples ^a | | | Positive samples (%) | Concentration range ^b (µg L ⁻¹) |
|------------------|------------------------------------|--------------------------|------------------------|----------------------|--|
| | < 0.1 µg L ⁻¹ | > 0.1 µg L ⁻¹ | > 1 µg L ⁻¹ | | |
| Herbicides | | | | | |
| Propazine | 44 | 0 | 0 | 44 | 0.021-0.031 |
| Terbuthylazine | 52 | 36 | 8 | 96 | 0.007-19.552 |
| Simazine | 24 | 12 | 0 | 36 | 0.016-0.114 |
| Atrazine | 44 | 0 | 0 | 44 | 0.021-0.079 |
| <i>DET</i> | 76 | 12 | 8 | 96 | 0.011-1.385 |
| <i>DIA</i> | 16 | 4 | 0 | 20 | 0.024-0.111 |
| <i>DEA</i> | 0 | 0 | 0 | 0 | not detected |
| Metribuzin | 4 | 0 | 0 | 4 | 0.026 |
| Metamitron | 0 | 0 | 0 | 0 | not detected |
| Lenacil | 8 | 0 | 4 | 12 | 0.033-5.078 |
| Fluometuron | 56 | 28 | 8 | 92 | 0.026-8.512 |
| Metobromuron | 0 | 0 | 0 | 0 | not detected |
| Alachlor | 0 | 0 | 0 | 0 | not detected |
| Acetochlor | 4 | 0 | 0 | 4 | 0.049 |
| Metolachlor | 92 | 0 | 0 | 92 | 0.01-0.034 |
| Ethofumesate | 88 | 0 | 0 | 88 | 0.015-0.028 |
| Diclofop-methyl | 0 | 0 | 0 | 0 | not detected |
| Terbutryn | 28 | 4 | 0 | 32 | 0.035-0.671 |
| Fungicides | | | | | |
| Pyrimethanil | 80 | 0 | 0 | 80 | 0.003-0.083 |
| Cyprodinil | 96 | 0 | 0 | 96 | 0.012-0.030 |
| Metalaxyl | 64 | 4 | 4 | 72 | 0.003-2.263 |
| <i>CGA 92370</i> | 16 | 0 | 4 | 20 | 0.040-3.919 |
| Penconazole | 40 | 4 | 0 | 44 | 0.006-0.972 |
| Myclobutanil | 12 | 8 | 0 | 20 | 0.021-0.268 |

| | | | | | |
|-----------------|----|----|---|----|--------------|
| Triadimenol | 16 | 16 | 4 | 36 | 0.020-1.659 |
| Flutriafol | 16 | 4 | 0 | 20 | 0.009-0.100 |
| Tebuconazole | 88 | 4 | 0 | 92 | 0.018-0.166 |
| Kresoxim-methyl | 92 | 0 | 0 | 92 | 0.020-0.033 |
| Nuarimol | 0 | 4 | 0 | 4 | 0.106 |
| Benalaxyl | 12 | 4 | 0 | 16 | 0.007-0.046 |
| Insecticides | | | | | |
| Dimethoate | 0 | 0 | 0 | 0 | not detected |
| Pirimicarb | 40 | 0 | 0 | 40 | 0.023-0.038 |
| Diazinon | 4 | 4 | 0 | 8 | 0.054-0.135 |
| Chlorpyrifos | 0 | 0 | 0 | 0 | not detected |

^a The term '<0.1 ug/L' referred to 'less than 0.1 ug/L' but greater than LOD

^b Number of replicates was two, each replicate injected twice.

Table 4. Concentration of pesticides found ($\mu\text{g L}^{-1}$) in three real groundwater samples after SPE with Oasis HLB and LiChrolut EN cartridges.

| Compound | Sample 2 | | Sample 10 | | Sample 25 | |
|-----------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| | LiChrolut | Oasis | LiChrolut | Oasis | LiChrolut | Oasis |
| Fluometuron | 1.1 \pm 0.2 | 1.2 \pm 0.2 | 0.061 \pm 0.009 | 0.058 \pm 0.007 | 8 \pm 1 | 8 \pm 1 |
| DET | 0.49 \pm 0.06 | 0.51 \pm 0.07 | 0.011 \pm 0.005 | 0.017 \pm 0.003 | 1.1 \pm 0.1 | 1.1 \pm 0.1 |
| DIA | 0.019 \pm 0.004 | 0.027 \pm 0.006 | - | - | 0.10 \pm 0.01 | 0.11 \pm 0.01 |
| Propazine | - | - | 0.031 \pm 0.004 | 0.028 \pm 0.005 | - | - |
| Atrazine | 0.05 \pm 0.01 | 0.07 \pm 0.01 | - | - | 0.036 \pm 0.006 | 0.024 \pm 0.008 |
| Terbuthylazine | 0.40 \pm 0.08 | 0.37 \pm 0.05 | 0.19 \pm 0.03 | 0.20 \pm 0.03 | 19 \pm 3 | 19 \pm 2 |
| Simazine | 0.10 \pm 0.01 | 0.11 \pm 0.01 | 0.025 \pm 0.004 | 0.024 \pm 0.003 | 0.12 \pm 0.02 | 0.11 \pm 0.01 |
| Pyrimethanil | - | - | 0.010 \pm 0.003 | 0.013 \pm 0.002 | 0.014 \pm 0.004 | 0.011 \pm 0.003 |
| Pirimicarb | 0.028 \pm 0.004 | 0.033 \pm 0.006 | 0.028 \pm 0.008 | 0.028 \pm 0.005 | 0.038 \pm 0.008 | 0.029 \pm 0.007 |
| Metalaxyl | 0.043 \pm 0.006 | 0.049 \pm 0.007 | 0.008 \pm 0.003 | 0.007 \pm 0.002 | 0.024 \pm 0.004 | 0.019 \pm 0.003 |
| Metolachlor | 0.031 \pm 0.004 | 0.018 \pm 0.007 | - | 0.017 \pm 0.005 | 0.034 \pm 0.006 | 0.020 \pm 0.005 |
| Terbutryn | 0.029 \pm 0.006 | 0.038 \pm 0.005 | - | - | 0.035 \pm 0.007 | 0.039 \pm 0.005 |
| Ethofumesate | 0.048 \pm 0.007 | 0.021 \pm 0.005 | - | - | 0.015 \pm 0.004 | 0.016 \pm 0.002 |
| Penconazole | 0.046 \pm 0.008 | 0.037 \pm 0.006 | 0.027 \pm 0.005 | - | 0.021 \pm 0.004 | 0.017 \pm 0.003 |
| Triadimenol | 0.10 \pm 0.01 | 0.13 \pm 0.02 | 0.020 \pm 0.009 | 0.021 \pm 0.006 | 0.067 \pm 0.009 | 0.059 \pm 0.007 |
| Cyprodinil | 0.057 \pm 0.008 | 0.019 \pm 0.007 | 0.040 \pm 0.006 | 0.019 \pm 0.005 | 0.020 \pm 0.003 | 0.014 \pm 0.004 |
| Myclobutanil | 0.14 \pm 0.02 | 0.13 \pm 0.02 | - | - | - | - |
| Kresoxim-methyl | - | - | 0.024 \pm 0.005 | 0.022 \pm 0.003 | 0.031 \pm 0.006 | 0.022 \pm 0.004 |
| Benalaxyl | 0.004 \pm 0.003 | 0.007 \pm 0.004 | - | - | - | - |
| Tebuconazole | 0.07 \pm 0.01 | 0.073 \pm 0.009 | 0.033 \pm 0.006 | 0.018 \pm 0.004 | 0.040 \pm 0.006 | 0.037 \pm 0.007 |

4 CONCLUDING REMARKS

A sensitive multi-residue analytical method based on SPE followed by GC–MS was developed for the simultaneous analysis of 34 of the most commonly used agricultural pesticides and degradation products in groundwater in the vineyard region of La Rioja (Spain). The highest recoveries in the SPE procedure were achieved when 500 mL of water were preconcentrated using an Oasis HLB cartridge, and acetonitrile and acetone were used as elution solvents. Matrix-matched standards were used to eliminate the variable matrix effect. Calibration curves showed good linearity in the range of concentrations studied, and low LODs (1–37 ng L⁻¹) were achieved for most of the selected compounds, allowing their detection at the levels established by EU legislation. The method developed was applied to the analysis of 25 real groundwater samples taken from vineyard areas in La Rioja. Analysis revealed the presence of several herbicides, such as terbuthylazine and its degradation compounds DET and DIA, fluometuron and metolachlor. Several fungicides were detected, such as metalaxyl and its metabolite CGA-92370, penconazole, tebuconazole, and kresoxim-methyl. Some of these herbicides and fungicides were detected in concentrations higher than the EU limit, even reaching values ten times higher than this value. Insecticides were the least common pesticides found, and this was consistent with their scarce use in La Rioja. Only pirimicarb and diazinon were detected in some of the samples analyzed, but their concentrations were in general lower than 0.1 µg mL⁻¹ in all the samples and never reached values as high as fungicides and herbicides.

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Occurrence of pesticides and some of their degradation products in waters in a Spanish wine region

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Abstract

A multi-residual analytical method based on solid phase extraction (SPE) followed by liquid chromatography-electrospray ionization-mass spectrometry (LC-MS) was developed to monitor pesticides in natural waters. Fifty-eight compounds, including herbicides, fungicides, insecticides and some of their degradation products, were surveyed to evaluate the quality of natural waters throughout the wine-growing region of La Rioja (Rioja DOCa). Ninety-two sampling points were selected, including surface and ground waters that could be affected by agricultural activities covering the region's three sub-areas. Different parameters that may affect the efficiency of the SPE procedure were optimized (sorbent type, elution solvent and sample volume), and matrix-matched standards were used to eliminate the variable matrix effect and ensure good quantification. The developed method determines the target compounds below the level established by the European Union for waters for human use with suitable precision (relative standard deviations lower than 18 %) and accuracy (with recoveries over 61 %). Forty compounds included in this study (six insecticides, twelve herbicides, sixteen fungicides and six degradation products) were detected in one or more samples. The herbicides terbutylazine, its metabolite desethyl terbutylazine, fluometuron and ethofumesate and the fungicides pyrimethanil and tebuconazole were the compounds most frequently detected in water samples (present in more than 60 % of the samples). Concentrations above 0.1 µg L⁻¹ were detected for thirty-seven of the compounds studied, and in several cases recorded values of over 18 µg L⁻¹. The results reveal the presence of pesticides in most of the samples investigated. In 64 % of groundwaters and 62 % of surface waters, the sum of compounds detected was higher than 0.5 µg L⁻¹ (the limit established by EU legislation for the sum of all pesticides detected in waters for human use).

Keywords: Multi-residue analysis, pesticides, surface and ground-waters, vineyards

1 INTRODUCTION

The use of pesticides plays an important role in harvest quality and food protection, providing enormous benefits in increasing production, as pests and diseases damage up to one-third of crops (Tadeo, 2008). As a result of massive global consumption (Sabik et al., 2000), pesticides and their degradation products spread through the environment and can contaminate water resources (Menezes Filho et al., 2010). Surface and especially ground waters located in intensive agricultural areas are more vulnerable to pesticide contamination, which is a major concern if the water is intended for human consumption.

This uptake of pesticides into watercourses is now a topic of considerable environmental interest due to the increasing number of compounds detected and has required the establishment of strict

directives (Palma et al., 2009) by the European Commission (EC) to minimise the impact on the environment. Accordingly, the European Union has established different directives, such as the Water Framework Directive 2000/60/EC, whose main objective is to protect water quality (EC, 2000). In 2008, Directive No. 2008/105/EC was introduced, establishing a list of 33 priority substances to be controlled in water, with a third of the list being pesticides (EC, 2008).

Given the interest in water pollution, monitoring studies have been conducted in the USA (Monplaisir et al., 2010), in several countries in Europe, such as Hungary (Maloschik et al., 2007), France (Comoretto et al., 2007; Baran et al., 2008), Italy (Guzella et al., 2006), Greece (Vryzas et al., 2009), Portugal (Palma et al., 2009), Serbia (Dujakovic et al., 2010) and Spain (Carabias-Martinez et al., 2002; Belmonte Vega et al., 2005; Kuster et al., 2008; Postigo et al., 2010) and outside Europe, such as Egypt (Potter et al., 2007), Morocco (El Bakouri et al., 2008) and China (Xue et al., 2005) to evaluate pollution in surface and ground waters for future remediation, as appropriate.

Pesticide residues may reach the aquatic environment through nonpoint and point pollution sources by direct run-off or leaching of these compounds or by careless disposal of empty containers or the washing of equipment after their application. Although significant advances have been made in controlling point-source pollution, little progress has been made regarding the nonpoint-source pollution of natural waters due to the seasonality, inherent variability and multiplicity of origins of nonpoint-source pollution. Surface water contamination by pesticides usually depends on the farming season, while groundwater contamination has a stronger persistence, which may have continuous toxicological effects for human health if used for public consumption.

The pollution of surface and ground waters by pesticides is governed by the physicochemical characteristics of the compounds (solubility in water, their capacity to be retained by soil components and their degradation rate), the properties of the medium in which they are applied, their abiotic and biotic degradation (Barra-Caracciolo et al., 2010) and other external factors, such as local rainfall and wind patterns or the topology of the area (Carabias-Martinez et al., 2000; Árias-Estevez et al., 2008). Indicators of the potential risk of water pollution based on these pesticide properties, such as the GUS index (groundwater ubiquity score) (Gustafson, 1989) have been introduced to allow classifying pesticides into potential leachers ($GUS > 2.8$), non-leachers ($GUS < 1.8$) and transient leachers ($1.8 < GUS < 2.8$).

In large areas of Spain, the pollution due to pesticides used to increase agricultural production merits special attention. Concerning wine-growing specifically, a large number of pesticides belonging to different chemical classes are being used annually to combat weeds, insects or fungi (AEPLA, 2011). The number of pesticide treatments per year depends on the weather conditions. Wine-growing is the main agricultural activity in the La Rioja region (N. Spain), which is the fifth Spanish region with the highest investment per hectare in crop protection products, with a consumption of pesticides of 13.79 kg ha^{-1} in 2008 (MARM, 2011). Vines are grown over an area that accounts for 34 % of the region's total arable land (159,127 ha), and its importance is based on the considerable economic activity it generates (Rioja DOCa - Qualified Designation of Origin, 2011). Some of the soils in this region have low organic matter contents and could facilitate the pollution of groundwaters. Although most of the drinking water in La Rioja is provided by groundwater (Navarrete et al., 2008), there is a lack of monitoring data. Few studies have been carried out until now to evaluate pesticide residues in waters in the wine-growing region

referred to as the Rioja Qualified Designation of Origin (Rioja DOCa). Thus, monitoring studies are required to evaluate diffuse and point pollution due to the use of these compounds in agriculture or to identify historic pollution present in groundwaters for remediation purposes, if necessary. Some studies have been published reporting the presence of different pesticides along the River Ebro in that region (Quintana et al., 2001; Claver et al., 2006; Hildebrandt et al., 2008; Navarro et al., 2010), but the sampling points were too limited to obtain a complete assessment of the water condition in this area.

The aim of this work was to conduct a thorough monitoring of surface and ground waters to evaluate possible pollution by pesticides in a region with intensive agricultural activities, and mainly vineyard cultivation. The monitoring programme was undertaken to assess the occurrence of insecticides (10), herbicides (19) and fungicides (18) belonging to different chemical classes and widely used in the region of Rioja DOCa, as well as some of their degradation products (11). The spatial sampling network involved 92 vulnerable sites throughout the three different sub-areas in the region (Rioja Alavesa, Rioja Alta and Rioja Baja). Thirteen of these samples corresponded to surface water and seventy-nine to groundwater samples. A reliable multi-residue method based on solid-phase extraction (SPE) and liquid chromatography with mass spectrometry (LC-MS) was developed and optimised for determining and quantifying the pesticides in this monitoring programme according to the levels required by EU legislation.

2 MATERIALS AND METHODS

2.1 Chemicals

Standards of pesticides and some of their degradation products were purchased from Riedel-de Haën (Seelze-Hannover, Germany), Fluka and Dr. Ehrenstorfer (Augsburg, Germany), and were used without further purification (minimum purity higher than 98 %). The compounds studied, belonging to several chemical classes, are listed in Table 1, including their use and some of their physicochemical characteristics (Footprint, 2011). Stock standard solutions (1000 or 500 $\mu\text{g mL}^{-1}$) for each of the analytes were first prepared by dissolving standards of pesticides in methanol and then stored in the dark at 4 °C. An intermediate standard solution (10 $\mu\text{g mL}^{-1}$) was prepared by appropriate dilution of the stock solutions in methanol, and this mixture was used as spiking solution for the aqueous calibration standards.

Different types of sorbents: polymeric cartridges -Oasis HLB (60 mg, Waters), Strata X (60 mg, Phenomenex) and LiChrolut EN (200 mg, Merck)-, silica-based bonded C18 cartridges (Sep-Pak Plus 900 mg, Waters) and carbon cartridges (Samplic 500 mg, Agilent) were used to optimise the SPE procedure for analyte preconcentration.

The organic solvents, acetonitrile, methanol, hexane and acetone, were of HPLC grade and supplied by Fischer Scientific (Loughborough, UK), being used as received. Ultra-high quality (UHQ) water was obtained with a Milli-Q water purification system (Millipore, Milford, MA, USA). All other chemicals used were of analytical reagent grade.

Table 1. Common names, uses and physicochemical properties of pesticides selected for the study (data taken from Footprint, 2011) and conditions for analytical determination of pesticides and degradation products. Chemicals with letters in brackets correspond to degradation compounds of parent compounds with the same letter as superscripts.

| Pesticide | Field of use | Water Solubility (mg L ⁻¹) | GUS index | Log Kow | m/z | V cone (V) | SIM window | RT (min) |
|----------------------------------|--------------|--|--------------|---------|-------|---------------|---------------|----------|
| Chlorsulfuron | Herbicide | 12500 | 5.38 | -0.99 | 358.1 | 20 | 1 | 4.52 |
| CGA-62826 (a) | Degr. Prod. | - | - | | 266.2 | 25 | 1 | 4.8 |
| Flazasulfuron | Herbicide | 2100 | 2.34 | -0.06 | 408.2 | 20 | 1 | 5 |
| DIHA (b) | Degr. Prod. | - | - | - | 156.1 | 25 | 1 | 6.6 |
| Acephate | Insecticide | 790000 | 1.14 | -0.85 | 184.1 | 15 | 1 | 6.9 |
| DEHA (c) | Degr. Prod. | - | - | - | 170.1 | 25 | 1 | 7.3 |
| Cymoxanil | Fungicide | 780 | -0.37 | 0.67 | 199.2 | 35 | 1 | 8.3 |
| Pyrimidinol (d) | Degr. Prod. | - | - | | 153.2 | 25 | 1 | 8.8 |
| DIA (e) | Degr. Prod. | 670 | - | - | 174.2 | 25 | 1 | 9.3 |
| Imidacloprid | Insecticide | 610 | 3.76 | 0.57 | 256.2 | 15 | 1 | 9.3 |
| Chloridazon | Herbicide | 422 | 2.54 | 1.19 | 222.1 | 30 | 2 | 10.3 |
| Dimethoate | Insecticide | 39800 | 1.05 | 0.70 | 230.2 | 15 | 2 | 10.5 |
| Metamitron | Herbicide | 1770 | 3.09 | 0.85 | 203.2 | 25 | 2 | 10.6 |
| HA (f) | Degr. Prod. | - | - | - | 198.1 | 25 | 2 | 10.8 |
| DEA (g) | Degr. Prod. | 3200 | 3.54 | - | 188.1 | 25 | 2 | 11.1 |
| CGA 92370 (h) | Degr. Prod. | - | - | - | 194.2 | 25 | 2 | 11.5 |
| Carbendazim | Fungicide | 8.0 | 2.64 | 1.48 | 192.2 | 35 | 2 | 12.1 |
| Carbofuran | Insecticide | 322 | 3.02 | 1.8 | 222.2 | 20 | 2 | 12.3 |
| HT (i) | Degr. Prod. | 7.19 | 4.59 | | 212.2 | 25 | 3 | 12.9 |
| Metribuzin | Herbicide | 1165 | 2.57 | 1.65 | 215.1 | 20 | 3 | 13.2 |
| CMPU (j) | Degr. Prod. | - | - | | 185.1 | 20 | 3 | 13.5 |
| DET (k) | Degr. Prod. | 327.1 | 3.54 | | 202.2 | 20 | 3 | 13.5 |
| Carbaryl | Insecticide | 9.1 | 2.02 | 2.36 | 202.2 | 15 | 3 | 14.0 |
| Flutriafol | Fungicide | 95.0 | 5.29 | 2.3 | 302.2 | 20 | 3 | 14.1 |
| Lenacil | Herbicide | 2.9 | 4.25 | 1.69 | 235.2 | 15 | 3 | 14.9 |
| Isoproturon | Herbicide | 70.2 | 2.07 | 2.5 | 207.2 | 25 | 3 | 15.2 |
| Chlorotoluron ^j | Herbicide | 74 | 2.79 | 2.5 | 213.2 | 20 | 3 | 15.5 |
| Metalaxyl ^{a,h} | Fungicide | 8400 | 2.11 | 1.65 | 280.2 | 25 | 3 | 15.9 |
| Atrazine ^{b,c,e,f,g} | Herbicide | 35 | 3.30 | 2.7 | 216.1 | 30 | 3 | 16.0 |
| Metobromuron | Herbicide | 330 | 2.52 | 2.41 | 259.1 | 20 | 3 | 16.1 |
| Fluometuron | Herbicide | 111 | 3.92 | 2.28 | 233.2 | 20 | 3 | 16.2 |
| Pirimicarb | Insecticide | 3100 | 2.73 | 1.70 | 239.2 | 20 | 3 | 16.8 |
| Diuron | Herbicide | 35.6 | 1.83 | 2.87 | 233.2 | 25 | 3 | 17.0 |
| Nuarimol | Fungicide | 26 | 3.52 | 3.18 | 315.1 | 30 | 4 | 17.9 |
| Cyproconazole | Fungicide | 93 | 3.25 | 3.09 | 292.2 | 20 | 4 | 18.3 |
| Azoxystrobin | Fungicide | 6.0 | 2.53 | 2.5 | 404.2 | 25 | 4 | 18.3 |
| Propazine | Herbicide | 8.6 | 3.84 | 3.95 | 230.2 | 25 | 4 | 18.7 |
| Myclobutanil | Fungicide | 132 | 3.54 | 2.89 | 289.1 | 25 | 4 | 19.0 |
| Terbutylazine ^{b,e,i,k} | Herbicide | 6.6 | 3.07 | 3.4 | 230.2 | 25 | 4 | 19.1 |

| Pesticide | Field of use | Water Solubility (mg L ⁻¹) | GUS index | Log Kow | m/z | V cone (V) | SIM window | RT (min) |
|-----------------------|--------------|--|--------------|---------|-------|---------------|---------------|----------|
| Ethofumesate | Herbicide | 50 | 3.38 | 2.7 | 287.2 | 20 | 4 | 19.2 |
| Iprovalicarb | Fungicide | 17.8 | 2.35 | 3.2 | 321.3 | 20 | 5 | 19.9 |
| Triadimenol | Fungicide | 72.0 | 3.75 | 3.18 | 296.2 | 15 | 5 | 20.1 |
| Pyrimethanil | Fungicide | 121 | 2.65 | 2.84 | 200.2 | 35 | 5 | 20.3 |
| Methoxyfenozide | Insecticide | 3.3 | 3.02 | 3.72 | 369.3 | 20 | 5 | 20.5 |
| Linuron | Herbicide | 63.8 | 2.03 | 3.0 | 250.1 | 20 | 5 | 20.6 |
| Dimethomorph | Fungicide | 28.95 | 2.56 | 2.68 | 388.2 | 25 | 5 | 20.6 |
| Acetochlor | Herbicide | 282 | 1.77 | 4.14 | 270.2 | 15 | 5 | 22.1 |
| Metolachlor | Herbicide | 530 | 3.49 | 3.4 | 284.2 | 15 | 5 | 22.3 |
| Penconazole | Fungicide | 73.0 | 1.51 | 3.72 | 284.2 | 20 | 6 | 23.5 |
| Kresoxim-methyl | Fungicide | 2.0 | 1.82 | 3.4 | 314.4 | 15 | 6 | 24.4 |
| Tebuconazole | Fungicide | 36.0 | 2.0 | 3.7 | 308 | 25 | 6 | 24.6 |
| Benalaxyl | Fungicide | 28.6 | 0.51 | 3.54 | 326.2 | 25 | 6 | 25.2 |
| Cyprodinil | Fungicide | 13 | 1.2 | 4.0 | 226.2 | 40 | 6 | 25.5 |
| Diazinon ^d | Insecticide | 60 | 1.14 | 3.69 | 305.2 | 20 | 6 | 25.8 |
| Trifloxystrobin | Fungicide | 0.61 | 0.53 | 4.5 | 409.1 | 20 | 6 | 26.9 |
| Dichlofop-methyl | Herbicide | 0.39 | 0 | 4.8 | 341.2 | 20 | 6 | 27.3 |
| Chlorpyrifos | Insecticide | 1.05 | 0.15 | 4.7 | 350.1 | 20 | 6 | 27.6 |
| Cypermethrin | Insecticide | 0.009 | -2.12 | 5.3 | 416.2 | 20 | 6 | 28.6 |

2.2 Apparatus and chromatographic conditions

Liquid chromatography with mass spectrometric detection (LC–MS) was carried out using a Waters (Milford, MA, USA) system equipped with a model e2695 multisolvent delivery and autosampler system coupled with a Micromass-ZQ single quadrupole mass spectrometer detector with an ESI interface and Empower software as the data acquisition and processing system. The MS parameters were as follows: capillary voltage, 3.1 kV; source temperature, 120 °C; the cone and desolvation temperatures were set at 20 and 300 °C, respectively; the desolvation gas flow was set at 400 L h⁻¹ and the cone gas flow at 60 L h⁻¹.

The compounds were separated in a 150 mm × 4.60 mm Luna PFP2 analytical column, packed with 3.0 µm particles (Phenomenex, Torrance, CA, USA) with a C-18 Waters Sentry pre-column (Waters, Milford, MA, USA). The mobile phase was consisted of methanol (solvent A) – 5 mM ammonium formate at pH = 5 (solvent B). The elution gradient was as follows: the mobile phase started with 60 % of methanol, which was increased linearly to 75 % in 4 min, and kept constant for 3 min, then raised to 80 % in 4 min. and kept constant for 3 min. The percentage was then raised to 100 % in 6 min and kept constant for 5 min; finally, it was returned to the initial conditions in 2 min. The column was equilibrated for 5 min, and flow rate was 0.3 mL min⁻¹. The volume injected was 20 µL.

The operating conditions of the MS system were optimised in the scan mode (scan range, m/z 50-500). Quantification was performed by external calibration. Calibration curves were obtained by plotting analyte peak areas (obtained from the total ion chromatogram (TIC) in SIM mode) versus

concentration using matrix-matched standards (uncontaminated blank water samples spiked with standard analyte solutions, which were managed in a similar way to collected water samples). Several blank samples were performed during the extraction and injected every fifteen samples to check the presence of memory effects. And several standards were injected at different times of the sample set to check the goodness of the calibration.

2.3 Description of the study area

The Rioja DOCa wine region is located in northern Spain, straddling the River Ebro. Figure 1 shows a map of the area. The local terrain perfectly delimits the region and sets it apart from the surrounding area. Its 63,593 hectares of vineyards are divided between three provinces on the Upper Ebro - La Rioja (43,885 ha), Alava (12,934 ha) and Navarre (6,774 ha). One hundred kilometres separate Haro, the westernmost town, from Alfaro, the easternmost. The valley has a maximum width of about 40 km, being covered in vineyards that occupy successive terraces to an altitude of around 700 m above sea level. The whole area benefits from the confluence of two climates, Atlantic and Mediterranean, which provide mild temperatures and an annual rainfall of slightly above 400 L m⁻². The region itself is divided into three sub-areas: Rioja Alavesa, which is significantly influenced by the Atlantic climate, and its soils, in general, are chalky-clay in terraces and small plots; Rioja Alta, with the climate being also mainly Atlantic, while the soils are chalky-clay, ferrous-clay or alluvial and, finally, Rioja Baja, with a drier and warmer climate and alluvial and ferrous-clay soil types. In general, the soils have low organic matter content (<2 %), a sandy clay loam or sandy loam texture and moderate water content, favouring the mobility of pesticides (Rioja DOCa - Qualified Designation of Origin, 2011). Besides vineyards, the other crops in this area are cereals, fruit trees, sugar beet and potatoes (Government of La Rioja, 2006). Table 2 shows the main characteristics of the sampling sites, including the hydrogeological units or aquifers where the samples are located, the type of crops and the existence or not of irrigation in the surrounding areas that could influence the type and levels of pesticides detected. The number of wells and springs in Rioja Alta and Rioja Alavesa is higher than in Rioja Baja, where irrigation is provided by river water (Lodosa canal). However, in general, the wells in Rioja Baja are deeper than in the other two regions, where the water table can be just a few meters below the surface.

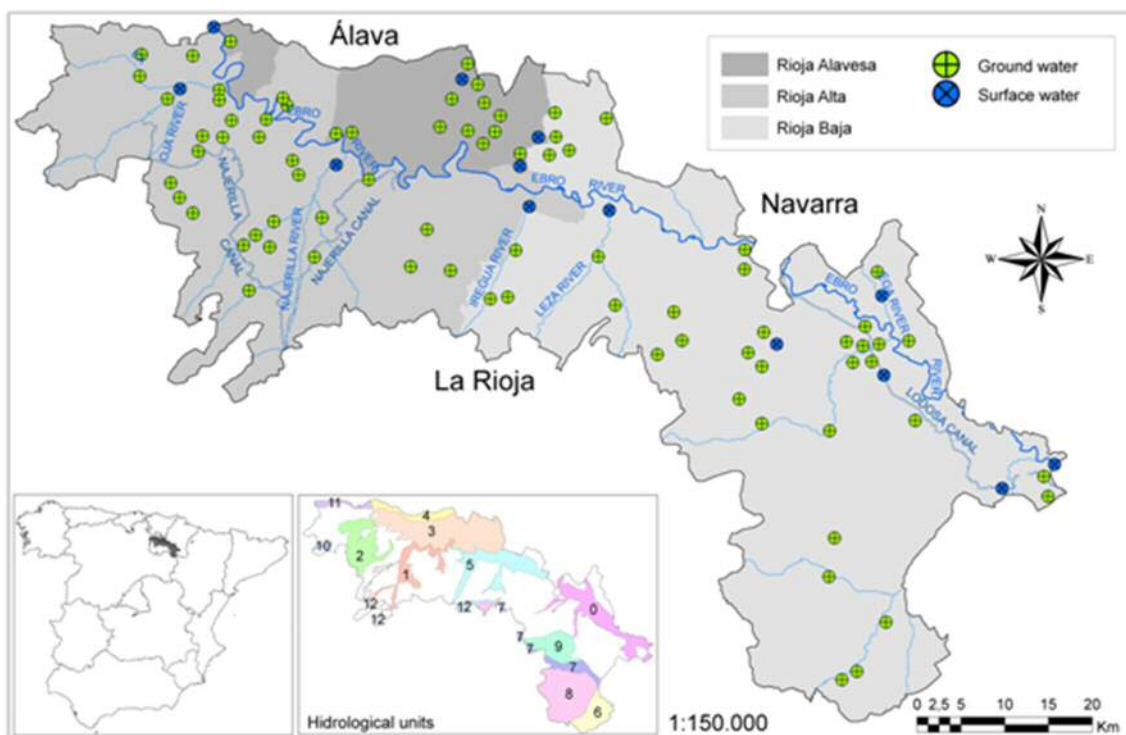


Figure 1. Map of the Rioja DOCa wine-growing region in Spain, indicating the sampling points studied. The names of the hydrological units corresponding to numbers are included in Table 2.

2.4 Sample collection

Water samples were collected in 2 L amber glass bottles and transported to the laboratory in iceboxes. Within four days, the samples were filtered through nitrocellulose screens with 0.45 µm pore size membranes (Millipore), being kept refrigerated at 4 °C in the dark prior to extraction. The extracts were analysed within two weeks of collection.

A total of ninety-two water samples were collected in March 2011 from different areas affected by agricultural development throughout the three different sub-areas of Rioja Alavesa (15 points), Rioja Alta (34 points) and Rioja Baja (43 points) (Figure 1 and Table 2). Thirteen of these samples corresponded to surface waters (two from the River Ebro at opposite ends of La Rioja region, six more from the main tributaries, two more from the Lodosa canal and three more from small rivers) and seventy-nine samples corresponded to groundwaters from privately dug wells with different depths varying between 1 and 15 m, in general, and public sources or springs. Only three samples came from depths of between 17 and 60 m (Table 2). The dug wells were located inside the cultivated fields or next to them, being generally used for irrigation purposes. Samples were collected manually or pumped, depending on the well type.

Table 2. Characteristics of the sampling points monitored in the three sub-areas studied in the La Rioja region.

| Sampling point | Hydrogeological unit or aquifer ^a | Water type | Water depth (m) | Characteristics of the area | |
|----------------|--|---------------|-----------------|------------------------------------|-----------|
| | | | | Crops cultivated | Watering |
| Rioja Alavesa | | | | | |
| ALV-G1 | Laguardia alluvial (3) | Groundwater | 1-2 | Vineyards | Yes |
| ALV-G2 | Laguardia alluvial (3) | Groundwater | 1-2 | Vineyards | Yes |
| ALV-G3 | Laguardia alluvial (3) | Groundwater | <5 | Cereals and vineyards | No |
| ALV-G4 | Laguardia alluvial (3) | Groundwater | Spring | Vineyards | No |
| ALV-G5 | Laguardia alluvial (3) | Groundwater | Spring | Vineyards | No |
| ALV-G6 | Laguardia alluvial (3) | Groundwater | Spring | Vineyards | No |
| ALV-G7 | Laguardia alluvial (3) | Groundwater | Spring | Vineyards and olives | No |
| ALV-G8 | Laguardia alluvial (3) | Groundwater | <5 | Vineyards | Yes |
| ALV-G9 | Laguardia alluvial (3) | Groundwater | Spring | Vineyards and cereals | No |
| ALV-G10 | Laguardia alluvial (3) | Groundwater | 3 | Vineyards | No |
| ALV-G11 | Laguardia alluvial (3) | Groundwater | <5 | Vineyards | No |
| ALV-G12 | Laguardia alluvial (3) | Groundwater | Spring | Vineyards and cereals | No |
| ALV-S1 | Moreda river | Surface water | - | Vineyards and olives | No |
| ALV-S2 | Oyón river | Surface water | - | Vineyards | - |
| ALV-S3 | Viñaspre river | Surface water | - | Vineyards and orchard | - |
| Rioja Alta | | | | | |
| ALT-G1 | Najerilla alluvial (1) | Groundwater | Spring | Vineyards and cereals | No |
| ALT-G2 | Najerilla alluvial (1) | Groundwater | Spring | Vineyards and cereals | No |
| ALT-G3 | Najerilla alluvial (1) | Groundwater | Spring | Vineyards and cereals | No |
| ALT-G4 | Najerilla alluvial (1) | Groundwater | Spring | Vineyards and cereals | No |
| ALT-G5 | Oja alluvial (2) | Groundwater | Spring | Cereals, vineyards and beet | No |
| ALT-G6 | Oja alluvial (2) | Groundwater | 8 | Cereals, vineyards and beet | No |
| ALT-G7 | Laguardia alluvial (3) | Groundwater | <5 | Vineyards, cereals and beet | No |
| ALT-G8 | Laguardia alluvial (3) | Groundwater | 2-3 | Vineyards and cereals | No |
| ALT-G9 | Oja alluvial (2) | Groundwater | Spring | Vineyards and cereals | No |
| ALT-G10 | Najerilla alluvial (1) | Groundwater | Spring | Vineyards | No |
| ALT-G11 | Oja-Conchas Haro (2,11) | Groundwater | 5-10 | Vineyards and cereals | No |
| ALT-G12 | Najerilla-Mendavia alluvial (1,5) | Groundwater | <5 | Vineyards and fruit trees | No |
| ALT-G13 | Oja alluvial (2) | Groundwater | Spring | Vineyards | No |
| ALT-G14 | Najerilla alluvial (1) | Groundwater | 5-10 | Vineyards and cereals | No |
| ALT-G15 | Najerilla alluvial (1) | Groundwater | <5 | Vineyards, cereals and fruit trees | Sometimes |
| ALT-G16 | Oja alluvial (2) | Groundwater | Spring | Cereals and vineyards | No |
| ALT-G17 | Najerilla alluvial (1) | Groundwater | Spring | Vineyards | No |
| ALT-G18 | Oja alluvial (2) | Groundwater | Spring | Vineyards and orchards | Si |
| ALT-G19 | Oja alluvial (2) | Groundwater | Spring | Vineyards and cereals | No |
| ALT-G20 | Oja alluvial (2) | Groundwater | <5 | Vineyards and cereals | Yes, drip |
| ALT-G21 | Oja alluvial (2) | Groundwater | Spring | Vineyards and orchards | Yes |
| ALT-G22 | Oja alluvial (2) | Groundwater | Spring | Vineyards and orchards | Yes |
| ALT-G23 | Sierra de Cantabria (4) | Groundwater | 5-10 | Vineyards | No |
| ALT-G24 | Oja alluvial (2) | Groundwater | Spring | Vineyards | No |
| ALT-G25 | Laguardia alluvial (3) | Groundwater | 3 | Vineyards | No |
| ALT-G26 | Laguardia alluvial (3) | Groundwater | 45 | Vineyards and orchards | No |
| ALT-G27 | Najerilla alluvial (1) | Groundwater | Spring | Vineyards and cereals | No |
| ALT-G28 | Najerilla alluvial (1) | Groundwater | Spring | Vineyards and orchards | No |
| ALT-G29 | Pancorbo-Conchas de Haro (11) | Groundwater | 5-6 | Vineyards | No |
| ALT-G30 | Oja alluvial (2) | Groundwater | Spring | Vineyards and cereals | No |
| ALT-G31 | Oja alluvial (2) | Groundwater | 2-3 | Vineyards, cereals and potatoes | Yes |
| ALT-S1 | Ebro river | Surface water | - | Vineyards, olive and fruits | |
| ALT-S2 | Najerilla river | Surface water | - | Vineyards | |
| ALT-S3 | Oja river | Surface water | - | Vineyards | |

| Sampling point | Hydrogeological unit or aquifer ^a | Water type | Water depth (m) | Characteristics of the area | |
|----------------|--|---------------|-----------------|-----------------------------------|-----------|
| | | | | Crops cultivated | Watering |
| Rioja Baja | | | | | |
| BJ-G1 | Lodosa-Tudela alluvial (0) | Groundwater | Spring | Vineyards | No |
| BJ-G2 | Añavieja-Valdegutur alluvial (6) | Groundwater | 60 | Vineyards | Yes, drip |
| BJ-G3 | Añavieja-Valdegutur alluvial (6) | Groundwater | Spring | Fruit trees and orchards | Yes |
| BJ-G4 | Mendavia alluvial (5) | Groundwater | Spring | Fruit trees | Yes |
| BJ-G5 | Mendavia alluvial (5) | Groundwater | <3 | Olive trees | Yes, drip |
| BJ-G6 | Mendavia alluvial (5) | Groundwater | 3-4 | Vineyards | No |
| BJ-G7 | Mendavia alluvial (5) | Groundwater | Spring | Fruit trees | No |
| BJ-G8 | Mendavia alluvial (5) | Groundwater | 2-3 | Vineyards | Yes, drip |
| BJ-G9 | Mendavia alluvial (5) | Groundwater | 3 | Vineyards | Yes, drip |
| BJ-G10 | Lodosa-Tudela alluvial (0) | Groundwater | 4-5 | Vineyards | Yes, drip |
| BJ-G11 | Lodosa-Tudela alluvial (0) | Groundwater | 5 | Vineyards and fruit trees | Yes |
| BJ-G12 | Lodosa-Tudela alluvial (0) | Groundwater | 3-4 | Vineyards | No |
| BJ-G13 | Detritico Arnedo (9) | Groundwater | <5 | Orchards | Yes |
| BJ-G14 | Lodosa-Tudela alluvial (0) | Groundwater | 5-6 | Vineyards and olives | No |
| BJ-G15 | Lodosa-Tudela alluvial (0) | Groundwater | Spring | Vineyards | Yes, drip |
| BJ-G16 | Laguardia alluvial (3) | Groundwater | 3-4 | Vineyards and cereals | Sometimes |
| BJ-G17 | Añavieja-Valdegutur (6) | Groundwater | 17 | Fruit trees and orchards | Yes |
| BJ-G18 | Lodosa-Tudela alluvial (0) | Groundwater | 7-9 | Vineyards and olives | Yes, drip |
| BJ-G19 | Lodosa-Tudela alluvial (0) | Groundwater | 8-10 | Vineyards and olives | Yes, drip |
| BJ-G20 | Lodosa-Tudela alluvial (0) | Groundwater | > 10 | Vineyards | No |
| BJ-G21 | Lodosa-Tudela alluvial (0) | Groundwater | > 10 | Olive trees | No |
| BJ-G22 | Lodosa-Tudela alluvial (0) | Groundwater | > 10 | Orchards | Yes |
| BJ-G23 | Mendavia-Lodosa alluvial (0,5) | Groundwater | Spring | Vineyards, cereals and olives | No |
| BJ-G24 | Fitero-Arnedillo (7) | Groundwater | 5-10 | Vineyards and cereals | No |
| BJ-G25 | Añavieja-Valdegutur alluvial (6) | Groundwater | 5-6 | Orchards | Yes |
| BJ-G26 | Fitero-Arnedillo (7) | Groundwater | Spring | Vineyards and cereals | No |
| BJ-G27 | Mendavia Alluvial (5) | Groundwater | Spring | Vineyards, cereals and olives | No |
| BJ-G28 | Mendavia-Lodosa alluvial (0,5) | Groundwater | <5 | Vineyards | No |
| BJ-G29 | Lodosa-Tudela alluvial (0) | Groundwater | 3-4 | Vineyards | Yes |
| BJ-G30 | Lodosa-Tudela alluvial (0) | Groundwater | Spring | Vineyards | No |
| BJ-G31 | Mendavia alluvial (5) | Groundwater | 3-4 | Vineyards, cereals, fruit trees | No |
| BJ-G32 | Lodosa-Tudela alluvial (0) | Groundwater | 6-8 | Vineyards | No |
| BJ-G33 | Laguardia alluvial (3) | Groundwater | Spring | Vineyards and olives | No |
| BJ-G34 | Laguardia alluvial (3) | Groundwater | 6-8 | Vineyards | No |
| BJ-G35 | Laguardia alluvial (3) | Groundwater | 5-6 | Vineyards | Yes, drip |
| BJ-G36 | Lodosa-Tudela alluvial (0) | Groundwater | 8-10 | Vineyards and cereals | Yes |
| BJ-S1 | Lodosa canal | Surface water | - | Vineyards, olives and fruit trees | |
| BJ-S2 | Lodosa canal | Surface water | - | Vineyards and fruit trees | |
| BJ-S3 | Ebro river | Surface water | - | Vineyards | |
| BJ-S4 | Ega river | Surface water | - | Vineyards | |
| BJ-S5 | Iregua river | Surface water | - | Vineyards | |
| BJ-S6 | Leza river | Surface water | - | Vineyards | |
| BJ-S7 | Villar de Arnedo river | Surface water | - | Vineyards | |

^a Numbers in parenthesis correspond to distribution of hydrological units in Figure 1

2.5 Sample preparation

Water samples were preconcentrated by SPE on a Waters extraction manifold (Milford, MA, USA) passing a volume of 500 mL through the Oasis HLB cartridges with a Gilson Minipuls 2 HP 8 peristaltic pump at a flow rate of 7 mL min⁻¹. Each cartridge was conditioned with 5 mL of acetone, 5 mL

of acetonitrile and 5 mL of UHQ water. After the adsorption of pesticides, the cartridges were dried in an air current under a vacuum of -20 mmHg for 5 min. The components retained were eluted with 4 mL of acetonitrile and then 4 mL of acetone. The organic phase obtained was evaporated to dryness under a nitrogen stream at 45 °C in an EVA-EC2-L evaporator (VLM GmbH, Bielefeld, Germany), and the dry residues obtained were re-dissolved in 0.5 mL of a 1:1 (v/v) methanol-water solution. The final extracts were filtered through 0.45 µm GHP Acrodisc filters (Waters Corporation) into LC vials and analysed.

3 RESULTS AND DISCUSSION

For this research, preliminary collection of available data was carried out to determine which pesticides have been recently used in the selected area. Fifty-eight compounds of different chemical classes and a wide range of physicochemical properties were selected according to data provided by public bodies, plant protection product dealers and local farmers. Some of the compounds and their main degradation products included in the list of 33 priority substances established by the EU to be controlled (EC, 2008) were also included (Table 1).

3.1 Optimisation of LC-MS method and SPE procedure

To optimise the MS conditions, experiments were carried out by direct infusion in the mobile phase of a standard solution of 10 µg mL⁻¹ of each target compound, operating the instrument under full scan. The solutions were prepared in methanol, and injected into the ESI source in positive mode at a range of cone voltages, and at a flow rate of 15 µg L⁻¹ min⁻¹. The cone voltage was studied for each compound in the 10–50 V range, with the cone voltage recording the largest peak area being chosen for quantification (Table 1). Identification of the target compound in unknown samples was based on the selection of the molecular ion and, in the cases where the compound studied contains Cl or Br, the relation of their isotopic masses was also used, because molecular ions should maintain these relations.

With a view to obtaining a more sensitive method for the quantification of the selected pesticides, a study was performed using SPE for sample enrichment, which is a pre-requisite for reaching detection limits below the legally established figure of 0.1 µg L⁻¹. Different parameters that may affect extraction efficiency were studied, namely, the SPE sorbent, the elution solvent and sample volume. Five cartridges were tested as SPE sorbent: three polymeric phases (Oasis HLB, LiChrolut EN and Strata-X), a modified silica-based material (C18), and a carbon-based material (SampliQ Carbon). Figure 2 shows the distribution of the recoveries obtained with different SPE cartridges when 100 mL of UHQ water spiked with all the analytes at a concentration of 1 µg L⁻¹ was passed through the cartridges.

The highest proportion of recoveries (>70 %) was obtained with the polymeric sorbent Oasis HLB (58 % of the compounds studied). For this reason, the Oasis HLB was selected as the best sorbent for preconcentrating water samples. Five solvents were tested to elute the retained analytes, methanol, acetonitrile, acetone, hexane and ethyl acetate, obtaining the best recoveries when a mixture of 4 mL of acetonitrile + 4 mL of acetone as elution solvent was used to elute the Oasis HLB cartridges. Under these conditions, more than 90 % of the analytes studied recorded recoveries higher than 65 %.

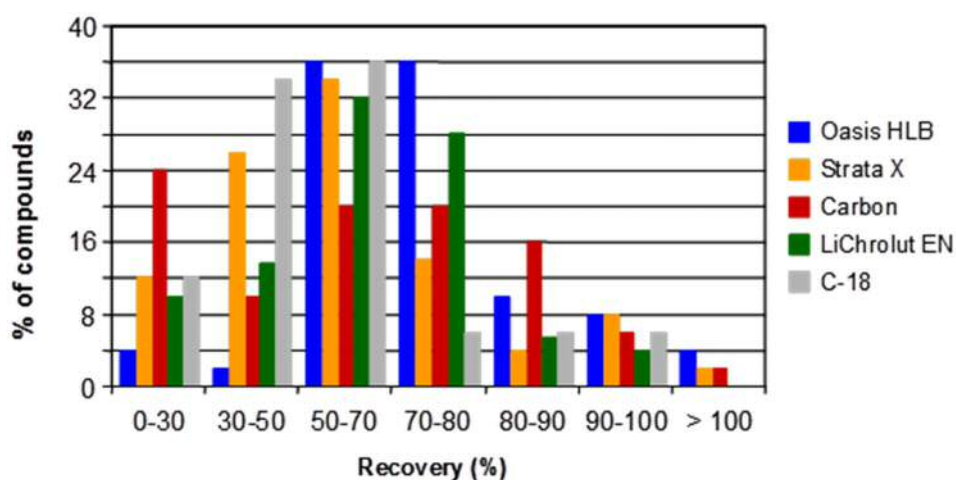


Figure 2. Distribution of recoveries for pesticides after solid phase extraction with different sorbents. Sample: 100 mL of UHQ water spiked with all the compounds at 5 µg L⁻¹, cartridges were eluted with methanol.

Finally, the influence of the sample volume to be preconcentrated was studied by loading different volumes (50-1000 mL) of water spiked with the same amount (0.2 µg) of each analyte. The overall results reveal no significant influence of the volume of water preconcentrated for most of the analytes studied. Only for pyrimidinol, metamitron, metribuzin and hydroxylated metabolites of atrazine, there was a small reduction in the recoveries when volumes higher than 250 mL were used and this reduction was further higher for volumes of 1000 mL. Therefore, a volume of 500 mL was selected as a compromise between the most satisfactory recovery values and the lowest detection limits that it was possible to obtain.

3.2 Method performance and validation

Although interferences are not visible in the LC–MS (SIM mode) chromatograms, co-eluting matrix components could inhibit or enhance the analyte signal, which can severely compromise the quantitative analysis of environmental samples. To evaluate the influence of the matrix on the analysis, 500 mL of three different kinds of water samples (UHQ, river and groundwater, previously analysed to verify the absence of any of the compounds studied) were spiked with the same amount (0.2 µg L⁻¹) of all the compounds selected. After extraction, the signals obtained for surface and ground water were compared with those of the UHQ water. In groundwater, 63 % of the compound studied recorded very low signal suppression or enhancement (-15 % to +15 %), and 23 % of the compounds presented a signal variation of < 25 %. Strong matrix effects were observed only for 14 % of the compounds studied, with chlorsulfuron, flazasulfuron, metribuzin and acetochlor being the compounds with the highest influence. Similar results were observed in surface water, although in this case the percentage of compounds with a very low effect was 68 %. Thus, to offset the matrix effect and avoid any under/over estimation of pesticides, a matrix-matched calibration in groundwater was used considering the largest number of samples of this kind.

The proposed methodology was validated for each of the compounds by studying the accuracy (average recovery) and precision (reproducibility and repeatability) at the level of concentration established by EU legislation and the limits of detection (LOD) and quantification (LOQ) of the complete

method. The accuracy and precision of the proposed method was determined by recovery experiments and the relative standard deviations (RSD) of the signals or peak areas obtained for each analyte corresponding to five groundwater samples spiked with $0.1 \mu\text{g L}^{-1}$ for each analyte. The recovery values were between 61 and 103 % with RSD in the 5-18 % range, except for dichlofop-methyl and cypermethrin, which could not be quantified at this level (Table 3).

To estimate linearity, LOD and LOQ, eight matrix-matched standards were prepared with 500 mL of groundwater (previously analysed to verify the absence of the pesticides and metabolites) spiked with all the analytes in the $0.1\text{--}2.0 \mu\text{g L}^{-1}$ concentration range. Good linear relationships were obtained with regression coefficients higher than 0.99 for all the compounds in the range studied (Table 3). The LODs and LOQs were estimated as the analyte concentration with a signal-to-noise-ratio of 3 and 10, respectively; the LODs ranged between 10 and 98 ng L^{-1} for metalaxyl and dichlofop-methyl, respectively; the LOQs ranged between 24 and 215 ng L^{-1} for metribuzin and dichlofop-methyl, respectively. Only flazasulfuron, deisopropylhydroxyatrazine (DIHA), deethylhydroxyatrazine (DEHA), acephate, cypermethrin and dichlofop-methyl had a higher LOQ than the limit established by the EU for drinking water (Table 3). The pesticide levels in the groundwater samples analysed were quantified by external standard calibration.

Table 3. Quality control parameters of the SPE-LC-MS method applied to the analysis of pesticides in surface and ground waters. Chemicals with letters in brackets correspond to degradation compounds of parent compounds with the same letter as superscripts.

| Pesticides | SIM ion (m/z) | Recovery ^a (%) | RSD (%) | r^2 ($0.1\text{--}2.0 \mu\text{g L}^{-1}$) ^b | LOD ^c ($\mu\text{g L}^{-1}$) | LOQ ^d ($\mu\text{g L}^{-1}$) |
|-----------------|------------------|------------------------------|------------|--|--|--|
| Chlorsulfuron | 358.1 | 67 | 11 | 0.990 | 0.025 | 0.071 |
| CGA-62826 (a) | 266.2 | 69 | 5 | 0.990 | 0.024 | 0.054 |
| Flazasulfuron | 408.2 | 71 | 12 | 0.993 | 0.041 | 0.106 |
| DIHA (b) | 156.1 | 63 | 14 | 0.991 | 0.046 | 0.112 |
| Acephate | 184.1 | 68 | 10 | 0.990 | 0.064 | 0.147 |
| DEHA (c) | 170.1 | 67 | 15 | 0.996 | 0.048 | 0.107 |
| Cymoxanil | 199.2 | 76 | 13 | 0.991 | 0.022 | 0.069 |
| Pyrimidinol (d) | 153.2 | 61 | 15 | 0.998 | 0.011 | 0.036 |
| DIA (e) | 174.2 | 82 | 8 | 0.999 | 0.013 | 0.034 |
| Imidacloprid | 256.2 | 103 | 7 | 0.991 | 0.019 | 0.048 |
| Chloridazon | 222.1 | 86 | 14 | 0.993 | 0.021 | 0.058 |
| Dimethoate | 230.2 | 80 | 18 | 0.996 | 0.023 | 0.054 |
| Metamitron | 203.2 | 69 | 16 | 0.990 | 0.019 | 0.030 |
| HA (f) | 198.1 | 71 | 10 | 0.997 | 0.037 | 0.065 |
| DEA (g) | 188.1 | 81 | 15 | 0.997 | 0.021 | 0.064 |
| CGA 92370 (h) | 194.2 | 68 | 8 | 0.997 | 0.021 | 0.067 |
| Carbendazim | 192.2 | 71 | 17 | 0.992 | 0.012 | 0.051 |
| Carbofuran | 222.2 | 93 | 12 | 0.993 | 0.026 | 0.071 |
| HT (i) | 212.2 | 76 | 11 | 0.990 | 0.027 | 0.080 |
| Metribuzin | 215.1 | 65 | 12 | 0.991 | 0.01 | 0.024 |

| Pesticides | SIM ion (m/z) | Recovery ^a (%) | RSD (%) | r ² (0.1-2.0 µg L ⁻¹) ^b | LOD ^c (µg L ⁻¹) | LOQ ^d (µg L ⁻¹) |
|-----------------------------------|------------------|------------------------------|------------|--|---|---|
| CMPU (j) | 185.1 | 90 | 10 | 0.994 | 0.039 | 0.089 |
| DET (k) | 202.2 | 84 | 9 | 0.994 | 0.016 | 0.046 |
| Carbaryl | 202.2 | 81 | 11 | 0.996 | 0.022 | 0.069 |
| Flutriafol | 302.2 | 91 | 7 | 0.996 | 0.023 | 0.062 |
| Lenacil | 235.2 | 94 | 5 | 0.996 | 0.026 | 0.061 |
| Isoproturon | 207.2 | 78 | 13 | 0.990 | 0.021 | 0.065 |
| Chlorotoluron ^j | 213.2 | 85 | 13 | 0.993 | 0.015 | 0.040 |
| Metalaxyl ^{a,h} | 280.2 | 86 | 8 | 0.997 | 0.010 | 0.032 |
| Atrazine ^{b,c,e,f,g} | 216.1 | 86 | 7 | 0.993 | 0.011 | 0.040 |
| Metobromuron | 259.1 | 77 | 5 | 0.996 | 0.018 | 0.061 |
| Fluometuron | 233.2 | 87 | 8 | 0.993 | 0.019 | 0.047 |
| Pirimicarb | 239.2 | 69 | 12 | 0.996 | 0.012 | 0.028 |
| Diuron | 233.2 | 93 | 10 | 0.992 | 0.013 | 0.041 |
| Nuarimol | 315.1 | 77 | 12 | 0.996 | 0.018 | 0.051 |
| Cyproconazole | 292.2 | 76 | 13 | 0.992 | 0.021 | 0.059 |
| Azoxystrobin | 404.2 | 85 | 11 | 0.995 | 0.017 | 0.052 |
| Propazine | 230.2 | 74 | 12 | 0.995 | 0.022 | 0.058 |
| Myclobutanil | 289.1 | 79 | 14 | 0.998 | 0.013 | 0.045 |
| Terbuthylazine ^{b,e,i,k} | 230.2 | 81 | 9 | 0.996 | 0.011 | 0.038 |
| Ethofumesate | 287.2 | 70 | 10 | 0.993 | 0.015 | 0.048 |
| Iprovalicarb | 321.3 | 86 | 7 | 0.995 | 0.016 | 0.037 |
| Triadimenol | 296.2 | 89 | 9 | 0.992 | 0.019 | 0.039 |
| Pyrimethanil | 200.2 | 63 | 16 | 0.992 | 0.014 | 0.038 |
| Methoxyfenozide | 369.3 | 73 | 10 | 0.998 | 0.016 | 0.042 |
| Linuron | 250.1 | 69 | 11 | 0.995 | 0.023 | 0.061 |
| Dimethomorph | 388.2 | 82 | 12 | 0.995 | 0.017 | 0.062 |
| Acetochlor | 270.2 | 80 | 15 | 0.995 | 0.023 | 0.054 |
| Metolachlor | 284.2 | 78 | 14 | 0.990 | 0.020 | 0.038 |
| Penconazole | 284.2 | 73 | 9 | 0.991 | 0.018 | 0.044 |
| Kresoxim-methyl | 314.4 | 76 | 11 | 0.995 | 0.019 | 0.036 |
| Tebuconazole | 308 | 77 | 10 | 0.991 | 0.012 | 0.035 |
| Benalaxyl | 326.2 | 89 | 15 | 0.992 | 0.015 | 0.048 |
| Cyprodinil | 226.2 | 73 | 9 | 0.994 | 0.018 | 0.043 |
| Diazinon ^d | 305.2 | 79 | 14 | 0.999 | 0.026 | 0.055 |
| Trifloxystrobin | 409.1 | 64 | 7 | 0.991 | 0.016 | 0.037 |
| Dichlofop-methyl | 341.2 | - | - | 0.990 | 0.098 | 0.215 |
| Chlorpyrifos | 350.1 | 65 | 12 | 0.991 | 0.031 | 0.055 |
| Cypermethrin | 416.2 | - | - | 0.991 | 0.094 | 0.207 |

^a Calculated from the replicated analysis (n = 5) of spiked (0.1 µg L⁻¹) groundwater samples; ^b Linear calibration range; ^c Limit of detection; ^d Limit of concentration that can be quantified

3.3 Pesticide monitoring in the studied area

The pesticide contamination of surface and ground waters in the Rioja DOCa wine-growing area was assessed by applying the developed and optimised method. Residual concentrations of both parent compounds and some of their degradation products were detected in most of the samples investigated. Out of the ninety-two points sampled, only one in Rioja Alta (ALT-G12) and three in Rioja Baja (BJ-G17, BJ-G23 and BJ-G36) did not contain any of the compounds studied possibly because the main agricultural activity in these points is not vineyard (Table 2), besides some of them are relatively deep wells.

Figure 3 shows the distribution of the concentrations of detected pesticides in all positive surface or ground waters, indicating the number of samples that exceed the quality standard of $0.1 \mu\text{g L}^{-1}$ for individual compounds. Figure 4 includes a distribution of the total samples for each pesticide according to the percentages of samples in which the pesticides were not detected or were detected in different concentration ranges: below the legally established limit ($0.1 \mu\text{g L}^{-1}$), between $0.1 \mu\text{g L}^{-1}$ and $1.0 \mu\text{g L}^{-1}$, and over $1.0 \mu\text{g L}^{-1}$.

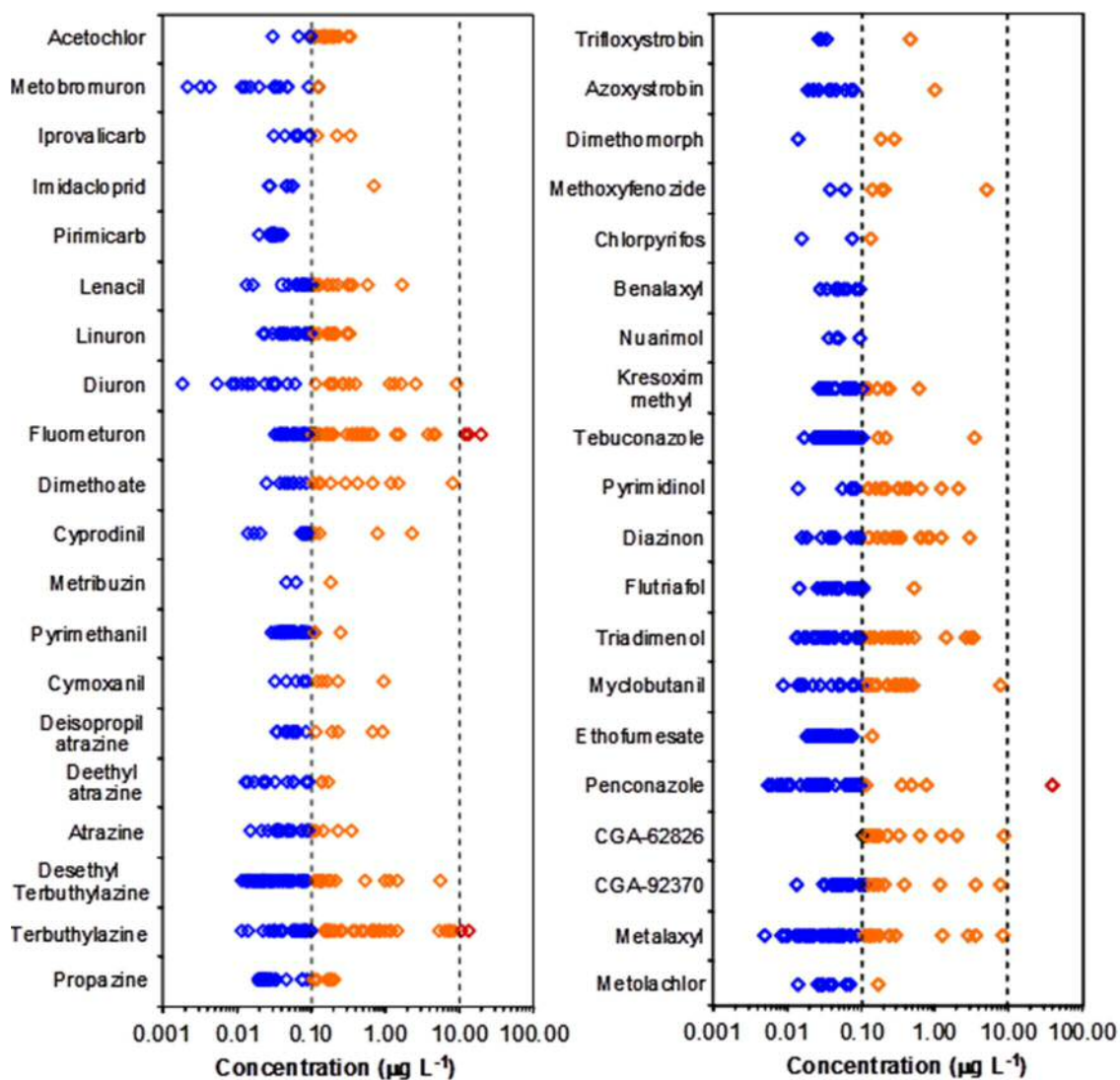


Figure 3. Concentrations of pesticides detected in the surface and groundwater samples (logarithmic scale).

Firstly, it is observed that forty compounds (six insecticides, twelve herbicides, sixteen fungicides and six degradation products) of the fifty-eight selected for this study (Table 3) were detected in one or more of the samples. However, some insecticides (acephate, carbaryl, carbofuran and cypermethrin) herbicides (metamitron, chloridazon, dichlofop-methyl, chlorotoluron and its metabolite CMPU, isoproturon, chlorsulfuron, flazasulfuron and the hydroxylated metabolites of triazines) and fungicides (cyproconazole and carbendazim) were not detected in any one of the samples analysed. Furthermore, the compounds pirimicarb, nuarimol and benalaxyl did not exceed the level of $0.1 \mu\text{g L}^{-1}$ in any sample, and other compounds such as pyrimethanil, metribuzin, imidacloprid, metobromuron, metolachlor, ethofumesate, flutriafol, chlorpyrifos, azoxystrobin and trifloxystrobin exceeded that level only in one sample. Other pesticides were detected at different concentration levels.

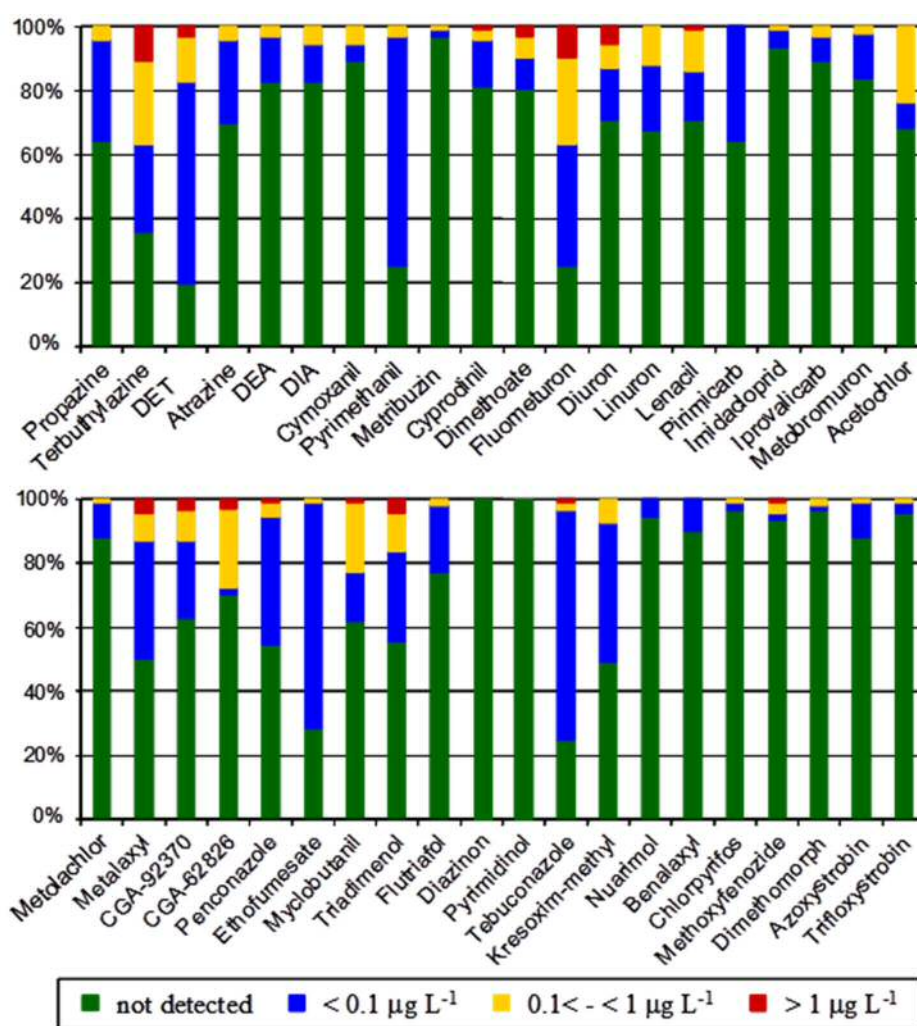


Figure 4. Distribution of samples with no detected concentrations of pesticides or detected concentrations below, over, or higher than ten times the legal limit for human consumption ($0.1 \mu\text{g L}^{-1}$).

The most ubiquitous compounds with the highest concentrations in all the samples were the herbicides terbuthylazine (67 % of the samples analysed, reaching values of $12.6 \mu\text{g L}^{-1}$), its metabolite desethylterbuthylazine (DET) (82 %, $5.2 \mu\text{g L}^{-1}$ of maximum concentration) and fluometuron (77 %, $18.7 \mu\text{g L}^{-1}$ of maximum concentration). These results are consistent with the widespread application of these herbicides in the area studied and their GUS index values; the latter, being between 3.07 and 3.92, which indicate these compounds are potential leachers (Footprint, 2011). Terbuthylazine has increased its use in recent years in place of atrazine, and has been detected in groundwaters in areas of Spain (Claver et al., 2006; Hildebrandt et al., 2008; Postigo et al., 2010) and Portugal (Gonçalves et al., 2007). However, atrazine concentrations in these earlier works were always higher than terbuthylazine concentrations, contrary to the results obtained here (Figure 3) possibly due to the use of atrazine was banned in the EU in 2004 and finally retired from the market in Spain and Portugal in 2007 (Decision 2004/248/CE). Fluometuron is not frequently monitored in groundwaters and there are no data on its presence. The high concentrations detected in this work could be due to the simultaneous application of fluometuron with terbuthylazine; in fact, the concentrations of both herbicides were significantly correlated when all the samples were considered ($r = 0.59$, $p < 0.001$).

The herbicides ethofumesate and diuron were detected in a large number of samples (72 %), albeit in lower concentrations ($0.133 \mu\text{g L}^{-1}$ of maximum concentration) or in a lower number of samples (29 %), but in a higher concentration ($8.551 \mu\text{g L}^{-1}$ of maximum concentration). Ethofumesate is used in beet cultivation and is classified as a potential leacher, with a GUS index of 3.38, and is also found at low concentrations in waters in other countries (Vryzas et al., 2009). Diuron is classified as a non-leacher (GUS index = 1.83), but it has also been detected in groundwaters when it is monitored (Claver et al., 2006; Postigo et al., 2010). Palma et al. (2009) considered this herbicide as one of the most abundant and ubiquitous compounds in surface waters in Portugal, together with atrazine, simazine, and terbuthylazine. The presence of herbicides in groundwaters at concentrations of $>10 \mu\text{g L}^{-1}$ has been reported (Carabias et al., 2000; Sanchez-Camazano et al., 2005; Gonçalves et al., 2007). This presence may be the result of intensive farming in the area and the extensive application of these compounds at the time of year when these samples were collected.

The herbicides atrazine and propazine were also detected in several samples (~ 40 %) even though their use was banned several years ago. The number of samples exceeding the limit of $0.1 \mu\text{g L}^{-1}$ was small (only four samples in each case, with a maximum concentration of $0.194 \mu\text{g L}^{-1}$ for propazine and $0.333 \mu\text{g L}^{-1}$ for atrazine). In addition, the presence of the degradation products deethylatrazine (DEA) and deisopropylatrazine (DIA) in nearly 20 % of the samples confirms contamination due to the use of these herbicides. Triazines and their degradation products have been found in groundwaters in many areas of Spain (Garrido et al., 2000; Carabias et al., 2000; Sánchez-Camazano et al., 2005; Postigo et al., 2010), as well as in other countries such as Greece (Papadopoulou-Mourkidou et al., 2004; Vryzas et al., 2009) and Portugal (Gonçalves et al., 2007; Palma et al., 2009). In this survey, the levels found for these compounds are lower than those previously reported, in keeping with the current ban on their use.

Fungicides were also detected in a large number of samples in different concentrations: pyrimethanil (75 % of samples and $0.234 \mu\text{g L}^{-1}$ of maximum concentration), tebuconazole (75 % and $3.236 \mu\text{g L}^{-1}$ of maximum concentration), kresoxim-methyl (51 % and $0.574 \mu\text{g L}^{-1}$ of maximum

concentration), metalaxyl (50 % and $8.015 \mu\text{g L}^{-1}$ of maximum concentration), penconazole (46 % and $18.72 \mu\text{g L}^{-1}$ of maximum concentration) and triadimenol (45 % and $3.103 \mu\text{g L}^{-1}$ of maximum concentration). Two degradation products of metalaxyl (CGA-62826 and CGA-92370) were also found in more than 30 % of the samples studied, with recorded concentration levels of more than $1 \mu\text{g L}^{-1}$ in three of the samples analysed. These compounds were identified in surface and ground waters in high concentrations, although they could be considered non-leachers according to the GUS index, which varies between 1.51 and 2.65 (with the exception of triadimenol). The occurrence of fungicides may be caused by their constant use in the vineyard areas where the sampling points were situated and by their increased use in a wide variety of vegetable, fruit, and grain crops. Few studies have investigated the presence of fungicides in surface and ground waters, although they have been found in different concentration ranges in several agricultural regions in Greece, Germany and the USA (Papadopoulou-Mourkidou et al., 2004; Berenzen et al., 2005; Battaglin et al., 2011), with the main compounds detected being azoxystrobin, metalaxyl, propiconazole, myclobutanil and tebuconazole.

The insecticides included in the study were found in a much smaller number of samples. This is consistent with the fact that these kinds of pesticides are used less in the areas studied. Pirimicarb and diazinon were found in fewer than 40 % of the samples, dimethoate in fewer than 20 % and chlorpyrifos and methoxyfenozide in fewer than 5 %. The metabolite of diazinon (pyrimidinol) was also found in 20 % of the samples. Some of these compounds with very high water solubility, such as dimethoate (39800 mg L^{-1}), or with a high GUS index, such as methoxyfenozide (3.02), were found in concentrations over the EU's legal limit. The concentrations of diazinon were $>0.1 \mu\text{g L}^{-1}$ in fewer than 20 % of the samples, and this insecticide is considered a non-leacher by the GUS index (1.14), but it is frequently found in ground waters (Postigo et al., 2010) or surface ones (Palma et al., 2009), generally below the limit of $0.1 \mu\text{g L}^{-1}$, but for long periods due to the wide use of this organophosphorus insecticide on different crops.

3.4 Pesticide distribution in the three different sub-areas in Rioja DOCa

The spatial distribution of these pesticides detected in the three different sub-areas in Rioja DOCa (Rioja Alavesa, Rioja Alta and Rioja Baja) was also studied, and the average and maximum concentrations of pesticides together with their detection frequency, reflected as the percentage of positive samples below and over the legal limit, are included in Table 4.

Several compounds and some degradation products (Table 4) were detected in most of the groundwaters (75 samples) and in all the surface waters (13 samples). Additionally, the sum of compounds detected was higher than $0.5 \mu\text{g L}^{-1}$ (the limit established by EU legislation for the sum of all pesticides detected in water for human consumption) in forty-eight groundwaters (64 % of total), corresponding to all the samples from Rioja Alavesa (Laguardia alluvial aquifer), except ALV-G12; twenty samples from Rioja Alta (mainly Najerilla alluvial and Oja alluvial aquifers) and seventeen samples from Rioja Baja (mainly the Mendavia alluvial and Lodosa-Tudela alluvial aquifers). In the rest of the groundwaters analysed (twenty-seven samples representing 36 % of the total), the presence of some pesticides was detected, but with a lower concentration. Again, one or more pesticides were detected in concentrations over the limit of $0.1 \mu\text{g L}^{-1}$ in nine of these samples (ALV-G12, ALT-G28 and -G30, BJ-G13, -G24, -G25, -G28, -G31 and -G33). The samples with lower pollution and no pesticides over the EU

limit corresponded largely to springs located in Rioja Alta (ALT-G3, -G4, -G5, -G7, -G9, -G16, -G22, -G23), and Rioja Baja (BJ-G2, -G3, -G7, -G8, -G15, -G18, -G26, -G27, -G34, -G35) (See Table 2 and Figure 1).

A negative although no significant relationship ($p>0.10$) was observed between the depth of the groundwater and the total content of pesticides. Wells deeper than 10 m recorded the lowest content of pesticides (e.g., BJ-G2 with a depth of 60 m and a total concentration of $0.137 \mu\text{g L}^{-1}$). On the other hand, groundwater samples from shallow wells (1-3 m) with the highest concentrations of pesticides corresponded, in general, to areas where the vineyard is the only crop, while less polluted groundwaters corresponded to wells located in areas where the main crops are fruit trees, cereals, etc. This is consistent with the higher amounts of pesticides used in vineyards for plant protection with regard to other crops. This explains the results obtained for the BJ-G17 sample collected from a well located in an area of mainly fruit trees and orchards, with no pollution detected, or the ALT-G26 sample, from a well 45 m deep, located in an area mainly of vineyards, where a very high concentration of pesticides ($4.58 \mu\text{g L}^{-1}$) was detected.

The sum of compounds detected in surface waters was higher than $0.5 \mu\text{g L}^{-1}$ in eight of the samples collected (62 % of the total): two samples from Rioja Alavesa, corresponding to small rivers in Oyón and Viñaspre, three samples from Rioja Alta, located in the rivers Ebro, Najerilla and Oja, and three samples from Rioja Baja, corresponding to the Lodosa canal and the River Ebro. It is noted that the concentration of pesticides detected at the inflow of the River Ebro into La Rioja ($3.22 \mu\text{g L}^{-1}$ total in ALT-S1) decreases when it leaves this region ($2.49 \mu\text{g L}^{-1}$ total in BJ-S3), despite the high amount of pesticides detected in the groundwaters of Rioja DOCa. This is because the river's inflow is lower than the outflow because of the large contribution made by its tributaries in this region and the ensuing dilution effect. Furthermore, a different behaviour in surface water was observed, while the main tributaries from Rioja Alavesa and Rioja Alta record a total content of pesticides that exceeded the limit of $0.5 \mu\text{g L}^{-1}$, the tributaries from Rioja Baja did not exceed that limit.

Table 4. Percentage of positive samples (or samples with some compound detected) with concentrations below and over 0.1 $\mu\text{g L}^{-1}$ and average and maximum concentrations for the pesticides detected in the different areas of study

| Pesticide | Rioja Alavesa (n = 15) | | | | Rioja Alta (n = 34) | | | | Rioja Baja (n = 43) | | | |
|---------------|------------------------|---------|--|------------------|----------------------|---------|--|------------------|----------------------|---------|--|------------------|
| | Positive samples (%) | | Concentration ($\mu\text{g L}^{-1}$) | | Positive samples (%) | | Concentration ($\mu\text{g L}^{-1}$) | | Positive samples (%) | | Concentration ($\mu\text{g L}^{-1}$) | |
| | C < 0.1 | C > 0.1 | Average \pm SD | C _{max} | C < 0.1 | C > 0.1 | Average \pm SD | C _{max} | C < 0.1 | C > 0.1 | Average \pm SD | C _{max} |
| Propazine | 47 | 0 | 0.024 \pm 0.008 | 0.043 | 35 | 0 | 0.032 \pm 0.021 | 0.81 | 23 | 9 | 0.071 \pm 0.064 | 0.194 |
| Terbutylazine | 20 | 60 | 2.386 \pm 4.053 | 12.597 | 24 | 53 | 1.235 \pm 2.045 | 7.171 | 23 | 32 | 0.917 \pm 2.307 | 9.900 |
| DETbz | 67 | 20 | 0.289 \pm 0.486 | 1.385 | 68 | 15 | 0.049 \pm 0.051 | 0.203 | 58 | 19 | 0.220 \pm 0.897 | 5.192 |
| Atrazine | 20 | 7 | 0.092 \pm 0.085 | 0.214 | 26 | 3 | 0.053 \pm 0.028 | 0.110 | 28 | 5 | 0.076 \pm 0.082 | 0.333 |
| DEA | 7 | 7 | 0.075 \pm 0.077 | 0.130 | 12 | 3 | 0.065 \pm 0.057 | 0.156 | 19 | 2 | 0.053 \pm 0.040 | 0.132 |
| DIA | 27 | 0 | 0.214 \pm 0.364 | 0.864 | 9 | 6 | 0.095 \pm 0.071 | 0.215 | 9 | 5 | 0.170 \pm 0.237 | 0.642 |
| Cymoxanil | 0 | 0 | 0 | 0 | 15 | 6 | 0.189 \pm 0.315 | 0.900 | 0 | 7 | 0.162 \pm 0.054 | 0.219 |
| Pyrimethanil | 55 | 18 | 0.057 \pm 0.016 | 0.089 | 74 | 6 | 0.056 \pm 0.039 | 0.234 | 65 | 2 | 0.049 \pm 0.016 | 0.105 |
| Metribuzin | 0 | 7 | 0.170 | 0.170 | 6 | 0 | 0.051 \pm 0.011 | 0.059 | 0 | 0 | 0 | 0 |
| Cyprodinil | 13 | 0 | 0.080 \pm 0.005 | 0.084 | 15 | 12 | 0.381 \pm 0.692 | 2.133 | 14 | 0 | 0.055 \pm 0.030 | 0.084 |
| Dimethoate | 0 | 7 | 0.173 | 0.173 | 15 | 12 | 1.144 \pm 2.444 | 7.549 | 9 | 9 | 0.228 \pm 0.367 | 1.114 |
| Fluometuron | 60 | 40 | 0.999 \pm 3.246 | 12.717 | 41 | 41 | 0.382 \pm 0.744 | 3.599 | 28 | 33 | 1.631 \pm 4.156 | 18.365 |
| Diuron | 20 | 27 | 0.344 \pm 0.534 | 1.512 | 12 | 12 | 1.684 \pm 2.907 | 8.551 | 19 | 9 | 0.072 \pm 0.088 | 0.247 |
| Linuron | 20 | 7 | 0.080 \pm 0.035 | 0.118 | 32 | 6 | 0.077 \pm 0.043 | 0.165 | 12 | 19 | 0.145 \pm 0.104 | 0.301 |
| Lenacil | 20 | 33 | 0.307 \pm 0.537 | 1.612 | 18 | 9 | 0.099 \pm 0.082 | 0.303 | 12 | 12 | 0.172 \pm 0.152 | 0.541 |
| Pirimicarb | 47 | 0 | 0.030 \pm 0.004 | 0.037 | 38 | 0 | 0.029 \pm 0.004 | 0.039 | 30 | 0 | 0.030 \pm 0.003 | 0.036 |
| Imidacloprid | 0 | 0 | | | 3 | 3 | 0.350 \pm 0.433 | 0.656 | 9 | 0 | 0.037 \pm 0.013 | 0.052 |
| Iprovalicarb | 13 | 7 | 0.077 \pm 0.030 | 0.111 | 12 | 3 | 0.107 \pm 0.118 | 0.315 | 2 | 2 | 0.147 \pm 0.080 | 0.204 |
| Metobromuron | 13 | 0 | 0.022 \pm 0.015 | 0.032 | 6 | 0 | 0.052 \pm 0.048 | 0.086 | 16 | 2 | 0.036 \pm 0.035 | 0.116 |

| Pesticide | | Rioja Alavesa (n = 15) | | | | Rioja Alta (n = 34) | | | | Rioja Baja (n = 43) | | |
|-----------------|----|------------------------|-------------|-------|----|---------------------|-------------|--------|----|---------------------|-------------|-------|
| Acetochlor | 13 | 40 | 0.154±0.071 | 0.292 | 9 | 38 | 0.143±0.065 | 0.314 | 5 | 7 | 0.149±0.055 | 0.224 |
| Metolachlor | 20 | 0 | 0.032±0.006 | 0.039 | 12 | 0 | 0.039±0.020 | 0.068 | 7 | 2 | 0.065±0.068 | 0.163 |
| Metalaxyl | 47 | 13 | 0.536±1.139 | 3.396 | 41 | 15 | 0.204±0.610 | 2.707 | 30 | 12 | 0.500±1.876 | 8.015 |
| CGA 92370 | 20 | 27 | 1.567±2.784 | 7.266 | 26 | 12 | 0.068±0.042 | 0.164 | 23 | 9 | 0.163±0.281 | 1.089 |
| CG-62826 | 7 | 53 | 1.392±2.835 | 8.250 | 3 | 26 | 0.252±0.342 | 1.158 | 0 | 21 | 0.173±0.157 | 0.587 |
| Penconazole | 60 | 0 | 0.052±0.033 | 0.089 | 44 | 12 | 2.022±8.396 | 18.721 | 30 | 2 | 0.048±0.086 | 0.338 |
| Ethofumesate | 80 | 0 | 0.037±0.014 | 0.067 | 79 | 0 | 0.032±0.012 | 0.071 | 60 | 2 | 0.038±0.022 | 0.133 |
| Myclobutanil | 20 | 33 | 1.033±2.498 | 7.208 | 12 | 26 | 0.189±0.137 | 0.474 | 16 | 16 | 0.143±0.142 | 0.404 |
| Triadimenol | 33 | 13 | 0.573±0.965 | 2.476 | 26 | 15 | 0.295±0.754 | 2.889 | 28 | 19 | 0.279±0.679 | 3.103 |
| Flutriafol | 47 | 7 | 0.058±0.033 | 0.101 | 21 | 3 | 0.106±0.154 | 0.484 | 12 | 0 | 0.049±0.026 | 0.095 |
| Diazinon | 0 | 20 | 1.337±1.447 | 2.807 | 18 | 18 | 0.182±0.224 | 0.822 | 12 | 14 | 0.351±0.371 | 1.128 |
| Pyrimidinol | 0 | 13 | 1.081±1.221 | 1.944 | 12 | 15 | 0.228±0.351 | 1.148 | 5 | 14 | 0.293±0.189 | 0.622 |
| Tebuconazole | 87 | 7 | 0.270±0.854 | 3.236 | 76 | 0 | 0.042±0.019 | 0.093 | 63 | 5 | 0.053±0.040 | 0.205 |
| Kresoxim-methyl | 53 | 7 | 0.078±0.054 | 0.212 | 47 | 9 | 0.088±0.122 | 0.574 | 37 | 7 | 0.072±0.046 | 0.232 |
| Nuarimol | 13 | 0 | 0.071±0.025 | 0.089 | 3 | 0 | 0.034 | 0.034 | 5 | 0 | 0.045±0.003 | 0.048 |
| Benalaxyl | 13 | 0 | 0.037±0.007 | 0.043 | 9 | 0 | 0.063±0.017 | 0.081 | 9 | 0 | 0.054±0.027 | 0.091 |
| Chlorpyrifos | 0 | 0 | | | 6 | 3 | 0.072±0.056 | 0.128 | 0 | 0 | | |
| Methoxyfenozide | 7 | 20 | 1.309±2.332 | 4.806 | 3 | 3 | 0.084±0.068 | 0.132 | 0 | 0 | 0 | 0 |
| Dimethomorph | 0 | 13 | 0.222±0.069 | 0.271 | 0 | 0 | 0 | 0 | 2 | 0 | 0.013 | 0.013 |
| Azoxystrobin | 7 | 7 | 0.493±0.636 | 0.943 | 15 | 0 | 0.042±0.023 | 0.074 | 9 | 0 | 0.037±0.023 | 0.070 |
| Trifloxystrobin | | | | | 6 | 3 | 0.166±0.236 | 0.439 | 2 | 0 | 0.025 | 0.025 |

4 CONCLUSIONS

The analytical method proposed, based on SPE-LC-MS, was optimised for the simultaneous determination of fifty-eight pesticides, including some of their degradation products. The highest recoveries for the majority of pesticides in the SPE procedure were achieved using Oasis HLB cartridges and acetonitrile-acetone as elution solvents. The application of this method to the analysis of ninety-two surface and ground waters in the wine-growing region of La Rioja showed that forty compounds were detected in at least three of the samples analysed. Terbutylazine, its metabolite DET, fluometuron, pyrimethanil, ethofumesate and tebuconazole were the compounds most frequently detected, appearing in over 60 % of the samples analysed. Only four of the sampling points (4 %) did not feature any of the compounds studied, with these points corresponding to wells with depths over 10 m or natural springs. Another thirty-two samples (34 %) recorded a total content of pesticides below $0.5 \mu\text{g L}^{-1}$, which is the quality standard for the total presence of pesticides set by EU legislation. Four out of these thirty-two samples correspond to surface water, and all of them are in Rioja Baja. Despite the high amount of pesticides detected in groundwaters in La Rioja, the total amount of pesticides detected at the inflow of the River Ebro into La Rioja was higher than the amount of pesticides detected at the outflow, which was due to the large increase in the river's flow in this region and the ensuing dilution effect. The results obtained reveal the widespread presence of pesticides over the area under study. However, a study of the temporal variation in surface and ground water pollution should also be considered, taking into account the seasonal application of pesticides in vineyards and other crops in this area.

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Development of a procedure for multiresidue analysis of pesticides in vineyard soils and its application to real samples

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Abstract

A procedure for multiresidue analysis was developed for extraction and determination of seventeen pesticides, including herbicides, fungicides and insecticides, as well as certain degradation products, in vineyard soils from La Rioja region (Spain). Different solvents and mixtures were tested in spiked pesticide-free soils, and pesticides were comparatively evaluated by gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. Recoveries > 70%, with RSD values < 9%, were obtained when a mixture of methanol:acetone or a mixture of methanol:CaCl₂ 0.01M for most polar compounds was selected as extraction solvent. Method validation was accomplished with acceptable linearity ($r^2 \geq 0.987$) within concentration range of 0.005 – 1 $\mu\text{g mL}^{-1}$ corresponding to 1.667 – 333.4 $\mu\text{g kg}^{-1}$ and 0.835 – 167.1 $\mu\text{g kg}^{-1}$ for LC-MS and GC-MS, and detection limits < 0.4 $\mu\text{g kg}^{-1}$ for the compounds studied. The extraction method was applied to 17 real vineyard soil samples, with terbutylazine and its metabolite desethylterbutylazine being the most ubiquitous compounds, as they were detected in the 100 % of the soils analyzed. The presence of fungicides was also high, and the presence of insecticides was lower than other pesticides. The results confirm the usefulness of the optimized procedure for monitoring residues in vineyard soils.

Keywords: Degradation products, multiresidue analysis, pesticides, vineyard soils

1 INTRODUCTION

The use of pesticides in the agricultural sector has provided enormous benefits in terms of increased production and quality, as pests and diseases damage up to one-third of crops in the growth, harvest and storage phases. Soils under pesticide applications may receive a significant and constant quantity of these compounds, which can be retained by soil materials, undergo transformations into metabolites, or be transported by runoff or leaching into surface and ground water [1]. As a consequence, pesticides are frequently detected in soil, sediments and other environmental matrices, where the risk they may pose has to be controlled because of their impact on the nature and level of residues in the crops [2]. For this reason, the study of the capability of soil to retain, filter and degrade pesticides is important, and the determination of pesticide residues (as well as their degradation products) in soils is vital [3]

Social concern about the levels of pesticides in the environment, with the ongoing trend observed in current legislation to reduce the maximum residue levels allowed in a variety of matrices, is increasing the number of samples to be analysed, as well as prompting the need for their accurate determination at very low levels [4]. Hence it is also important to develop rugged and robust methods for determining these compounds at very low concentrations in such complex matrices.

Sample preparation and compound extraction is one of the most delicate steps in pesticide determination at trace levels in soils. Pesticide extraction from soils generally requires the use of techniques for extracting bound residues. Traditionally, the analysis of pesticides in soils has required liquid–solid extraction assisted by sonication with organic solvents [5,6], or by mechanical shaking [7,8], or the Soxhlet extraction method [9]. Several modern extraction techniques have been introduced, such as solid-phase microextraction [10], dispersive liquid-liquid microextraction [11], supercritical fluid extraction [12,13], accelerated solvent extraction (ASE), also known as pressurised liquid extraction (PLE) [14–16], microwave-assisted extraction [17,18], modified matrix solid-phase dispersion [19], and more recently, the QuEChERS method, which consists of acetonitrile extraction/partitioning and a dispersive solid phase extraction step as the clean-up [20,21]. Most of these techniques usually requires the use of an additional clean-up or preconcentration step by solid-phase extraction.

Many of the methods cited above require expensive, sophisticated equipment (as PLE or supercritical fluid extraction) and subsequent partition or chromatographic purification steps, which significantly lengthen the procedures involved and do not always allow optimal recoveries of all the pesticides tested [22]. Nevertheless, conventional methods are still routinely used in some laboratories because they are simple and have a lower cost per analysis, although they require time and intensive labour.

On the other hand, most of the papers related to the analysis of pesticides in soils determine groups of pesticides belonging to the same family, and a smaller number of approaches deal with the multiresidue analysis of pesticides in soils, which is the situation most often found in agricultural soils [8,14].

La Rioja is a region in northern Spain characterized by extensive agricultural practices, with vine as the main crop [www.magrama.gob.es]. Previous studies carried out in this area by our research group have reported the presence of a wide range of pesticides (mainly fungicides and herbicides) in surface and ground waters [23,24]. However, to date, there is clearly a lack of data regarding the presence of pesticides in soils throughout this region, and especially in the target area for our previous work. To our knowledge, only a few authors have analyzed pesticide residues in vineyard soils from a river basin in Galicia [25], Andalusia [26], and the Ebro river basin [16].

The aim of this work was as follows: (i) to optimize an extraction procedure for the most ubiquitous compounds previously detected in waters from vineyard soils in La Rioja, (ii) to develop a quick and easy method to analyze these compounds, and (iii) to apply the optimized methodology to real soil samples from this region, where our previous work recorded the presence of several pesticides in surface and ground waters. Accordingly, eight fungicides (metalaxyl, and its metabolite CGA-62826, pyrimethanil, tebuconazole, myclobutanil, kresoxim-methyl, triadimenol and flutriafol); seven herbicides (fluometuron, terbuthylazine, and its metabolites desethylterbuthylazine and hydroxyterbuthylazine, lenacil, ethofumesate and acetochlor), and two insecticides (methoxyfenozide and pirimicarb) were selected and determined by two optimized chromatographic methods, liquid chromatography (LC) and gas chromatography (GC) coupled to a mass spectrometry detector (MS). These selected compounds were determined in waters at concentrations higher than the required level of $0.1 \mu\text{g L}^{-1}$ by EU legislation [24].

2 MATERIALS AND METHODS

2.1 Chemicals

Analytical standards of pesticides with over 99% purity from Dr. Ehrenstorfer (Augsburg, Germany) and Riedel de Haën and Fluka (Seelze-Hannover, Germany) were supplied by Sigma-Aldrich Química SA (Madrid, Spain). Table 1 shows some of the characteristics of the seventeen compounds selected [<http://sitem.herts.ac.uk/aeru/footprint/en/index.htm>].

Individual stock solutions of each compound at a concentration of 1000 µg mL⁻¹ and another stock solution containing a mixture of all the pesticides at a concentration of 10 µg mL⁻¹ were prepared and were used for the preparation of working standard solutions, or solutions to spike the soil samples by serial dilutions with methanol.

All the chemicals (CaCl₂) and organic solvents (methanol (MeOH), acetonitrile (MeCN) and acetone (ACT)) were analytical grade reagents purchased from Fisher Chemical (United Kingdom). Ultrapure water (UHQ) was obtained from a Milli-Q water system (Millipore, Milford, MA, USA).

Table 1. Characteristics of pesticides

| Pesticide | Family (Field Use) | Water solubility (mg L ⁻¹) | GUS Index | Log Kow | K _{OC} | DT50 |
|------------------------------|-----------------------|--|--------------|------------|-----------------|------|
| Metalaxyl (META) | Phenylamide (F) | 7100 | 2.91 | 1.65 | 162 | 42 |
| CGA-62826 (CGA) | Phenylamide (D.P.) | - | 3.83 | - | 38 | 31.2 |
| Pyrimethanil (PYR) | Anilinopyrimidine (F) | 121 | 2.65 | 2.84 | 301 | 55 |
| Tebuconazole (TBC) | Triazole (F) | 36 | 2.00 | 3.70 | 769 | 63 |
| Myclobutanil (MYCL) | Triazole (F) | 132 | 3.54 | 2.89 | 517 | 560 |
| Kresoxim-methyl (KRX) | Strobilurin (F) | 2.0 | 1.82 | 3.40 | 308 | 16 |
| Triadimenol (TRIA) | Triazole(F) | 72 | 3.75 | 3.18 | 750 | 250 |
| Flutriafol (FLUT) | Triazole(F) | 95 | 5.29 | 2.30 | 205 | 1358 |
| Ethofumesate (ETHO) | Benzofuran (H) | 50 | 3.19 | 2.70 | 187 | 70 |
| Acetochlor (ACET) | Chloroacetamide (H) | 282 | 1.77 | 4.14 | 156 | 14 |
| Lenacil (LEN) | Uracil (H) | 2.9 | 4.25 | 1.69 | 165 | 179 |
| Fluometuron (FLUO) | Phenylurea (H) | 111 | 3.92 | 2.28 | 67.4 | 63.6 |
| Terbuthylazine (TBZ) | Triazine (H) | 6.6 | 3.07 | 3.40 | 231 | 75.1 |
| Desethylterbuthylazine (DET) | Triazine (D.P.) | 327.1 | 3.90 | 2.30 | 78 | 70.5 |
| Hydroxyterbuthylazine (HT) | Triazine (D.P.) | 7.19 | - | 4.59 | 187 | - |
| Methoxyfenozide (MTXF) | Diacylhydrazine (I) | 3.3 | 3.02 | 3.72 | 402 | 146 |
| Pirimicarb (PIRI) | Carbamate (I) | 3100 | 2.73 | 1.70 | 388 | 86 |

F: fungicide, D.P: Degradation Product, H: Herbicide, I: Insecticide

2.2 Soils

Uncontaminated soils were used for designing and validating the soil pesticide extraction procedure and analytical method. Two blank soils with different characteristics were collected from the surface horizon (0-20 cm). One soil was collected from IRNASA's Muñovela experimental farm (Salamanca, Spain), and another with similar characteristics to the vineyard soils in La Rioja was

collected from Valdenebro (Valladolid, Spain). The samples were air-dried and sieved (< 2 mm), and their characteristics were determined following standard analytical methods [27] (Table 2). Prior to use, they were tested to confirm the absence of detectable pesticide residues.

Table 2. Characteristics of blank soil and real soil samples

| Soils | pH | CaCO ₃ % | OM% | Sand % | Silt % | Clay % |
|----------------|------|---------------------|------|--------|--------|--------|
| Blank soils | | | | | | |
| Valdenebro | 7.8 | 27.3 | 1.85 | 37.4 | 23.6 | 39.0 |
| Muñovela | 6.2 | - | 0.85 | 74.5 | 13.7 | 11.8 |
| La Rioja soils | | | | | | |
| ALT-S1 | 7.60 | 11.1 | 1.55 | 47.1 | 19.3 | 33.7 |
| ALT-S2 | 8.00 | 33.4 | 1.05 | 49.7 | 19.9 | 30.4 |
| ALT-S3 | 8.03 | 24.9 | 1.01 | 58.6 | 13.6 | 27.9 |
| ALT-S4 | 7.86 | 20.3 | 1.31 | 58.2 | 16.4 | 25.4 |
| ALT-S5 | 7.99 | 51.0 | 0.94 | 67.0 | 11.9 | 21.1 |
| ALT-S6 | 7.82 | 34.7 | 1.94 | 76.9 | 9.35 | 13.7 |
| ALV-S7 | 7.83 | 35.6 | 1.16 | 45.4 | 19.3 | 35.3 |
| ALV-S8 | 7.99 | 12.9 | 1.36 | 34.6 | 29.7 | 35.7 |
| BJ-S9 | 8.08 | 24.1 | 1.21 | 72.6 | 11.8 | 15.6 |
| BJ-S10 | 7.60 | 24.1 | 1.21 | 76.9 | 8.99 | 11.4 |
| BJ-S11 | 7.93 | 7.45 | 1.81 | 74.8 | 10.8 | 14.4 |
| BJ-S12 | 7.43 | 25.8 | 1.98 | 50.2 | 18.2 | 31.6 |
| BJ-S13 | 7.76 | 32.2 | 0.44 | 50.7 | 38.4 | 10.9 |
| BJ-S14 | 7.80 | 50.7 | 0.25 | 14.6 | 42.4 | 43.0 |
| BJ-S15 | 8.10 | 21.9 | 1.58 | 78.3 | 12.2 | 9.48 |
| BJ-S16 | 7.95 | 10.2 | 2.75 | 59.8 | 13.4 | 26.8 |
| BJ-S17 | 7.50 | 10.1 | 1.98 | 60.6 | 19.1 | 20.3 |

2.3 Apparatus and Operating Conditions

LC–MS detection was carried out using a Waters (Milford, MA, USA) system equipped with a model e2695 multisolvent delivery and autosampler system coupled with a Micromass ZQ single quadrupole mass spectrometer detector with an electrospray ionization (ESI) interface, using Empower software as the data acquisition and processing system. The MS parameters were as follows: capillary voltage, 3.1 kV; source temperature, 120°C; desolvation temperatures and desolvation gas flow set at 300°C and 400 L h⁻¹, respectively, and cone gas flow set at 60 L h⁻¹.

Compounds were separated in a 150×4.60 mm Luna PFP2 fully porous silica analytical column packed with 3.0 µm particles (Phenomenex, Torrance, CA, USA) with a C-18 Waters Symmetry pre-column (Waters, Milford, MA, USA) to improve the performance of the column. The mobile phase was methanol (solvent A) – 5 mM ammonium formate at pH = 5 (solvent B). The elution gradient was as follows: the mobile phase started with 60% of methanol, which was increased linearly to 80% in 3 min, and kept constant for 6 min, then changed to 100% in 15 min. and kept constant for 4 min; being finally

returned to the initial conditions and kept constant for 4 min. The overall time of the chromatogram was 32 min. The flow rate was 0.3 mL min^{-1} and the volume injected was $20 \text{ }\mu\text{L}$. The operating conditions of the MS system were optimized in the scan mode (scan range, m/z 50-500).

GC-MS was conducted on a gas chromatograph Agilent 7890 coupled to a 5975 MSD Agilent mass spectrometer (Agilent Technologies, Wilmington, DE, USA). Chromatographic separation was performed on a $30 \text{ m} \times 0.25 \text{ mm I.D.}$, $0.25 \text{ }\mu\text{m}$ film thickness DB-17ms capillary column (J&W, Avondale, USA). The mass spectrometer was operated in the electron impact ionization mode with an ionizing energy of 70 eV, and electron multiplier voltage was set at 1700 V and dwell time at 10 msec. The source and the transfer line temperatures were adjusted to 230°C and 280°C , respectively.

Helium was used as the carrier gas at a flow rate of 1.5 mL min^{-1} , and the volume injected was $1 \text{ }\mu\text{L}$. The injector was operated in solvent vent mode at 60°C . The oven temperature was programmed as follows: from an initial value of 60°C . with an increment of 40°C/min to 100°C , then an increment of 20°C/min to 200° , a third ramp of 1°C/min to 210°C , followed by an increment of 5°C/min to 250°C , and finally, an increment of 10°C/min to 310°C , with a hold time of 2 min. The overall time of the chromatogram was 32 min. A delay of 3 min was used to prevent damage to the filament in the ion source. Three product ions were selected among the more abundant ones, and one was chosen for quantification. The selected ions were checked with retention time and required to comply with the relative intensity of the qualifier ions in the $\pm 10\%$ range.

Quantitative analysis was performed by external calibration. Calibration curves were obtained by plotting the peak areas of each compound obtained from the total ion chromatogram (TIC) in SIM mode versus concentration.

2.4 Pesticide multiresidue extraction procedure

With a view to optimizing the multiresidue extraction procedure, different individual solvents (methanol, acetone, acetonitrile, ethyl acetate and water) or mixtures of them (methanol: acetone (50:50), methanol: CaCl_2 0.01M (50:50) and methanol: water (50:50) at pH 4, 7, and 9) were tested. Triplicate samples of two blank soils (uncontaminated) (5 g) with different characteristics were spiked with a volume of methanol containing all the pesticides to obtain a concentration of 0.1 mg kg^{-1} (low dose, LD) corresponding to the doses of these compounds generally applied in different agronomic uses. The solvent was evaporated at room temperature, and the spiked soils were left for 24 h prior to extraction.

The multiresidue extraction procedure using the optimized solvent was applied to blank soils spiked with a volume of methanol containing all the pesticides to obtain a concentration of 1 mg kg^{-1} (high dose, HD) to verify the possible application of this extraction procedure in the event of the overuse of these compounds. This procedure was also applied to blank soils spiked with a volume of methanol containing all the pesticides at concentrations of 0.1 mg kg^{-1} and 1 mg kg^{-1} after 7, 15 and 30 days of sample spiking in order to study the influence of pesticide ageing time in the soil prior to extraction.

Pesticide residues from the spiked blank soils were extracted by a batch method, involving the agitation of 5 g soil samples with 10 mL of a suitable solvent for 24 hours in a thermostated camera at 20°C , followed by centrifugation at 3100 g for 15 minutes. The extracts (6 mL) were collected and evaporated under a nitrogen stream at 45°C in an EVA-EC2-L evaporator (VLM GmbH, Bielefeld,

Germany) until completely dry. When an aqueous phase was used (methanol: water and methanol:CaCl₂ 0.01M.) the use of SPE cartridges was necessary to remove the aqueous fraction. The cartridges used were OASIS HLB [23], and they were conditioned with 5 mL of acetone, 5 mL of acetonitrile and 5 mL of UHQ water. After the adsorption of pesticides, the cartridges were dried in an air current under a vacuum of -20 mmHg for 5 min. The components retained were eluted with 4 mL of acetonitrile and then 4 mL of acetone. The organic phase obtained was evaporated to dryness as indicated above. The dry residues obtained from the extraction were redissolved in 0.5 mL of methanol and filtered through 0.45-µm GHP Acrodisc filters (Water Corporation) into chromatography vials for GC analysis. An aliquot of this solution (0.1 mL) was diluted with water, obtaining a volume of 0.2 mL of methanol:water (50:50) solution for analysis by liquid chromatography-mass spectrometry (LC-MS). Blank soil extracts were also obtained for calibration curves. All experiments were carried out in triplicate

2.5 Method performance and validation

The accuracy (average recovery) and precision (reproducibility and repeatability) of the method were determined by recovery experiments in five uncontaminated soil samples spiked with 0.1 mg kg⁻¹ of each pesticide. The relative standard deviations (RSD) of the signals or peak areas were obtained for each analyte by LC-MS and GC-MS. The linearity of the calibration curves was evaluated for all the pesticides using eight standards prepared in blank soil extracts, and managed as collected soil samples to avoid any under/overestimation of pesticide concentration (matrix-matched calibration standards) [8,28]. The concentrations were in the 0.005-1 µg mL⁻¹ range (corresponding to 0.835-167.1 µg kg⁻¹ for GC-MS and 1.667-333.4 µg kg⁻¹ for LC-MS). The limits of detection (LOD) and quantification (LOQ) were estimated as the analyte concentration with a signal-to-noise-ratio of 3 and 10, respectively.

2.6 Multiresidue analysis of real soil samples

Seventeen soil samples located in the Rioja region (northern Spain) were collected in March 2012 from the three subareas into which this region is divided: Rioja Alavesa (ALV), Rioja Alta (ALT) and Rioja Baja (BJ). Sampling points were selected according to previous results on pesticide residues in local surface and ground waters [24]. All the soil samples were collected and analysed as it is indicated in section 2.2 (Table 2). They were transported in labelled plastic bags to the laboratory and stored in the dark at below -18°C until analysis. Pesticide residues were subsequently extracted from these real soil samples using the optimized multiresidue procedure. In all cases, samples were analyzed in triplicate.

3 RESULTS AND DISCUSSION

3.1 Optimization of LC-MS and GC-MS operating conditions

The LC-MS and GC-MS operating conditions were optimised to find the best analytical technique to determine the selected pesticides. It was based on methods developed by Herrero-Hernández et al. [23,24], although as the number of compounds included in this study was lower, the temperature ramps in GC-MS and the elution gradients in LC-MS were adjusted to improve the separation of the compounds during chromatographic separation. The identification of the target compound in unknown samples was based on the selection of the molecular ion, and when the compound studied contained Cl or

Br, the ratio of their isotopic masses was also used. In order to optimize the GC-MS conditions, experiments were performed using a methanol standard solution of $1 \mu\text{g mL}^{-1}$ of each target compound, operating the instrument under full scan. The target and reference ions for each compound were selected by comparing the spectrum obtained for each compound with the NIST library spectrum. Generally, the most abundant and/or characteristic ions were selected for identifying and quantifying the analytes.

Table S1 includes results on the conditions for the analytical determination of each pesticide and both techniques.

Both methods provided good separation and determination of pesticides with good sensitivity. However, the determination of all the pesticides selected was only possible by LC-MS as HT, CGA and methoxyfenozide were not detected by GC-MS.

Figure 1 includes LC-MS extracted chromatograms of a soil spiked with pesticides included in this work at 0.1 mg kg^{-1} .

3.2 Optimization of the extraction procedure

The recovery experiments on the studied pesticides were initially optimized in the two uncontaminated soils with different characteristics fortified at 0.1 mg kg^{-1} . Different individual solvents or mixtures were tested, and Figure S1 includes recoveries obtained for some compounds (representative of all the pesticides studied) from the Valdenebro soil determined by LC-MS and GC-MS. Non relevant differences were found between the recoveries obtained for all the compounds from uncontaminated Valdenebro soil and Muñozela soil (data not shown) with different textures and properties, indicating that the extraction of the pesticides studied was not affected in these soils. Soil properties such as organic matter (OM) and clay normally increase the binding of pesticides by soils and render their extraction more difficult [29]. These effects were not observed here possibly because the range of OM content in soils used for recoveries was not very wide although it was, in general, in the range found for the real soil samples studied. However, significant differences in recoveries were recorded with different organic solvents.

The results obtained for extractions indicated that recoveries were very low when water was used as extraction solvent, $< 50\%$ and $< 30\%$, in general, for LC-MS and GC-MS techniques. The lower recoveries were obtained for kresoxim and tebuconazole ($12\text{-}20\%$ by LC-MS or GC-MS) with low water solubility, and the higher recoveries were obtained for pesticides with higher water solubility, such as metalaxyl ($> 70\%$), and fluometuron ($> 100\%$) determined by LC-MS or GC-MS. The extractions with individual organic solvents indicated recoveries in general $> 100\%$ of all compounds when pesticides are determined by LC-MS, and in the $60\text{-}120\%$ range when pesticides are determined by GC-MS. Exceptions were found for HT and CGA, with recoveries $< 25\%$ with all extractants, and pirimicarb with recoveries $< 15\%$ with ethyl acetate. The higher recoveries were always found for triadimenol and methoxyfenozide by LC-MS, and for ethofumesate and fluometuron by GC-MS. An enhancement of signals up to $+50\%$ was observed for some pesticides and solvents when determined by LC-MS, although the suppression of signals was also found for HT and CGA. However, the suppression of signals was more frequently observed by GC-MS, and the determinations by this technique were, in general, $< 100\%$.

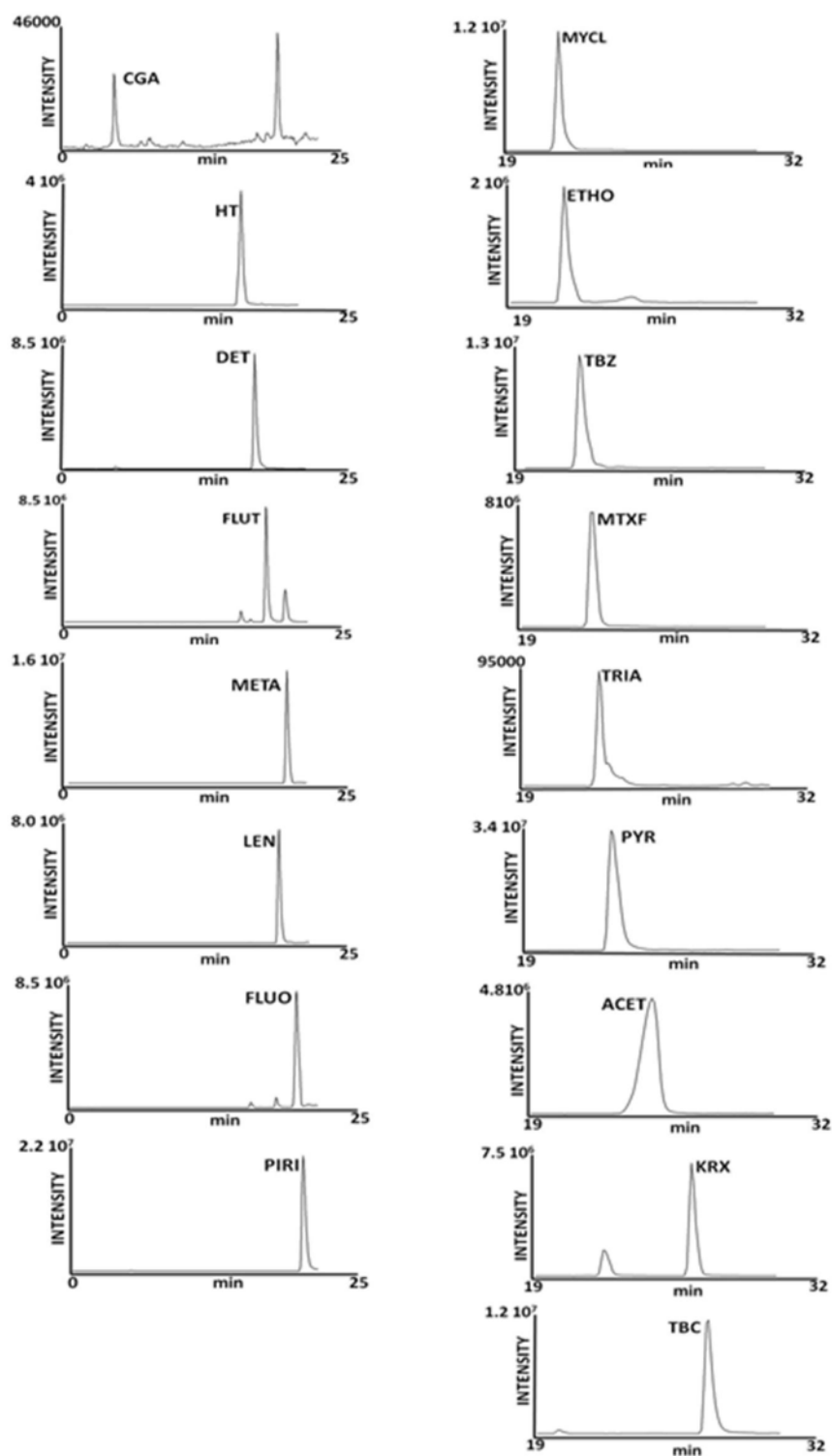


Figure 1. LC-MS extracted chromatograms of a soil spiked with pesticides included in this work at 0.1 mg kg^{-1} . The names of pesticides corresponding to abbreviations are included in Table 1.

The recoveries of all the pesticides were, in general, improved in mixtures of organic solvents, and in the methanol-acetone mixture (50:50) the recoveries were > 75% and > 70% for pesticides determined by LC-MS and GC-MS, respectively, excluding HT and CGA, neither of whose recoveries improved in these mixtures. The best recoveries for these compounds were obtained with mixtures of methanol:CaCl₂ 0.01M (50:50) (97.0 and 42.3%, respectively) or mixtures of methanol:water (50:50) at pH values of 7 and 9 (> 100%) for HT. The results are in a similar range to those reported by other authors using different extraction techniques for pesticides in soil [12,15].

The recoveries of pesticides extracted from soils after one day of spiking with the low dose (0.1 mg kg⁻¹), and ten times this dose (1 mg kg⁻¹) with the more suitable solvents, are included in Table 3, together with the recoveries of pesticides from spiked soils after different ageing times.

Table 3. Recoveries obtained in blank soil spiked at 0.1 mg kg⁻¹ (LD) and 1 mg kg⁻¹ (HD) after different times of pesticide in the soil

| Pesticide | Recovery % | | Recovery % | | Recovery % | | Recovery % | |
|-----------------|-----------------|-----------------|------------|-------|------------|-------|------------|-------|
| | 1 day | | 7 days | | 15 days | | 30 days | |
| | LD ^a | HD ^b | LD | HD | LD | HD | LD | HD |
| Metalaxyl | 90.1 | 102.2 | 90.1 | 128.1 | 100.2 | 113.2 | 125.3 | 109.3 |
| CGA-62826 | 42.3 | 48.3 | 40.3 | 39.6 | 35.2 | 37.8 | 38.4 | 36.2 |
| Pyrimethanil | 87.4 | 104.1 | 101 | 109.2 | 78.1 | 79.0 | 113.2 | 101.1 |
| Tebuconazole | 94.1 | 100.2 | 92.4 | 107.2 | 102.3 | 91.4 | 101.0 | 105.0 |
| Myclobutanil | 102.2 | 110.3 | 100.2 | 109.3 | 104.4 | 94.1 | 103.1 | 107.2 |
| Kresoxim-methyl | 99.1 | 104.1 | 88.3 | 118.4 | 92.2 | 97.0 | 112.4 | 101.3 |
| Triadimenol | 109.2 | 102.1 | 77.1 | 81.1 | 104.3 | 88.2 | 95.3 | 82.8 |
| Flutriafol | 112.4 | 107.2 | 88.2 | 113.2 | 98.2 | 102.3 | 110.2 | 95.3 |
| Ethofumesate | 98.7 | 105.3 | 124.1 | 113.4 | 74.1 | 87.6 | 41.5 | 32.7 |
| Acetochlor | 86.7 | 91.2 | 96.3 | 142.1 | 96.3 | 120.2 | 113.2 | 114.4 |
| Lenacil | 109.2 | 104.4 | 86.2 | 109.1 | 98.2 | 101.1 | 118.1 | 103.2 |
| Fluometuron | 82.1 | 98.1 | 108.1 | 118.2 | 118.1 | 113.4 | 115.2 | 108.1 |
| Terbutylazine | 86.3 | 103.3 | 84.2 | 105.3 | 94.2 | 94.6 | 89.2 | 76.9 |
| DET | 78.7 | 91.0 | 78.1 | 113.3 | 76.3 | 86.8 | 79.1 | 97.1 |
| HT | 97.0 | 98.5 | 90.3 | 88.51 | 95.2 | 92.7 | 72.8 | 80.0 |
| Methoxyfenozide | 98.4 | 106.2 | 90.3 | 123.2 | 102.4 | 112.1 | 127.2 | 123.2 |
| Pirimicarb | 93.3 | 101.1 | 81.2 | 80.2 | 90.3 | 92.4 | 92.5 | 94.6 |

LD^a, amount spiked 0.1 mg kg⁻¹; HD^b, amount spiked 1 mg kg⁻¹

The pesticides were determined only by the LC-MS method. The results indicate that, in general, the recoveries did not change for samples spiked with the high dose of pesticides after one day of spiking (the RSD of mean recoveries ranged between 1.09 and 12.7%). Similar results have been reported in the literature for other pesticides when different doses have been applied [7,12]. This tendency was maintained in samples after 7, 15 and 30 days of treatment. However, recoveries generally decreased after different pesticide ageing times in soil for samples spiked with a low or high dose, although some authors

have reported similar pesticide recoveries after different times of residence in the soil [12]. The more significant effects were seen for ethofumesate and HT. In the case of ethofumesate, the decrease was more pronounced, from 98.7 to 41.5% (samples with low dose), and from 105 to 32.7% (samples with high dose), indicating the possible formation of bound residues of this pesticide in the soil. Ethofumesate is a compound with a lower hydrophobic nature ($\log K_{ow} = 2.70$) than other pesticides studied, such as acetochlor, methoxyfenozide, kresoxim-methyl, tebuconazole, or terbuthylazine with K_{ow} value > 3 , although the sorption of this herbicide by soils and other organic sorbents has been reported in the literature [30,31].

3.3 Multiresidue method validation

The limits of detection (LOD), the limit of quantification (LOQ) and the linearity of the calibration curves were calculated. Values for LC-MS and GC-MS are shown in Table 4. Calibration curves were plotted for extracts of blank soil from Valdenebro to avoid the matrix effect. The range of the calibration curve was between 0.005 and 1 $\mu\text{g mL}^{-1}$, corresponding to a concentration range in soil of 1.667-333.4 $\mu\text{g kg}^{-1}$ for LC-MS and 0.835-167.1 $\mu\text{g kg}^{-1}$ for GC-MS, obtaining good results in both techniques for all the compounds, with values of r^2 of 0.987-0.997 for LC-MS and of 0.990-0.997 for GC-MS. The wide range of concentrations used in the calibration curve was due to the range of concentrations obtained for some pesticides in the real soil samples studied.

The LOD and LOQ were calculated at the lowest concentrations injected, yielding a signal/noise ratio of 3 and 10, respectively. The LOD values obtained for pesticides with LC-MS were between 0.066 and 0.334 $\mu\text{g kg}^{-1}$, and the LOQ values obtained were between 0.222 and 0.654 $\mu\text{g kg}^{-1}$. These values were higher for pesticides analyzed with GC-MS in the 0.156-0.387 $\mu\text{g kg}^{-1}$ range for the LOD and 0.263-0.510 for the LOQ. The LOD and LOQ values for all compounds recorded by the LC-MS analysis were lower than those obtained by GC-MS. In general, they were also below the maximum residue limits required by the EU in different agricultural products [http://ec.europa.eu/food/plant/protection/evaluation/database_act_subs_en.htm] so LC-MS provides greater selectivity for the determination of the pesticides studied in soils.

Table 4 also includes recoveries obtained for all the pesticides extracted from the Valdenebro soil spiked at 0.1 mg kg^{-1} with methanol-acetone or methanol- CaCl_2 (50:50), together with their RSD. The recovery values calculated ranged between 78.7 and 112.2% (LC-MS), and between 74.5 and 105.1% (GC-MS), and the RSD values ranged between 0.13 and 7.19 (LC-MS) and 3.02 and 8.97 (GC-MS), reflecting the high accuracy and precision of the extraction procedure tested. Furthermore, both analytical methods were proven to be effective for use in the analysis of pesticide multiresidues in soils, although it was evident that LC-MS allowed the determination of a larger number of compounds than GC-MS, and did so with greater precision.

Table 4. Validation parameters for determination of pesticide residues from blank soil by two analytical methods

| Pesticide | LC-MS | | | | | GC-MS | | | | |
|-----------------|---------------------------------------|-------------------------|-----------------|----------------------------|----------------------------|---------------------------------------|------------|-----------------|----------------------------|----------------------------|
| | Recovery % 0.1 mg kg ⁻¹ | RSD ^a n=5 | r ^{2b} | LOD µg kg ⁻¹ | LOQ µg kg ⁻¹ | Recovery % 0.1 mg kg ⁻¹ | RSD n=5 | r ^{2c} | LOD µg kg ⁻¹ | LOQ µg kg ⁻¹ |
| Metalaxyl | 90.1 | 1.94 | 0.990 | 0.066 | 0.221 | 102.1 | 3.99 | 0.993 | 0.160 | 0.264 |
| CGA-62826 | 42.3 | 4.52 | 0.990 | 0.217 | 0.504 | - | - | - | - | - |
| Pyrimethanil | 87.2 | 7.19 | 0.991 | 0.266 | 0.538 | 97.7 | 8.50 | 0.995 | 0.336 | 0.434 |
| Tebuconazole | 94.3 | 1.30 | 0.992 | 0.164 | 0.304 | 86.5 | 3.02 | 0.992 | 0.260 | 0.335 |
| Myclobutanil | 102.1 | 0.90 | 0.990 | 0.161 | 0.503 | 98.5 | 4.60 | 0.990 | 0.387 | 0.464 |
| Kresoxim-methyl | 99.1 | 0.14 | 0.997 | 0.303 | 0.654 | 92.5 | 4.31 | 0.994 | 0.378 | 0.510 |
| Triadimenol | 109.1 | 0.42 | 0.990 | 0.066 | 0.301 | 92.4 | 4.55 | 0.995 | 0.160 | 0.495 |
| Flutriafol | 112.2 | 0.40 | 0.987 | 0.066 | 0.222 | 89.2 | 5.30 | 0.994 | 0.156 | 0.264 |
| Ethofumesate | 98.7 | 2.56 | 0.989 | 0.334 | 0.504 | 105.1 | 5.71 | 0.993 | 0.256 | 0.423 |
| Acetochlor | 86.7 | 5.28 | 0.990 | 0.205 | 0.334 | 103.4 | 3.84 | 0.993 | 0.157 | 0.264 |
| Lenacil | 109.2 | 2.08 | 0.990 | 0.161 | 0.278 | 74.5 | 5.87 | 0.997 | 0.156 | 0.263 |
| Fluometuron | 82.1 | 2.62 | 0.996 | 0.066 | 0.302 | 87.7 | 8.97 | 0.991 | 0.328 | 0.425 |
| Terbuthylazine | 86.3 | 2.06 | 0.991 | 0.066 | 0.220 | 90.1 | 6.03 | 0.993 | 0.358 | 0.463 |
| DET | 78.7 | 0.28 | 0.994 | 0.161 | 0.305 | 91.1 | 7.13 | 0.992 | 0.156 | 0.264 |
| HT | 97.0 | 1.07 | 0.991 | 0.066 | 0.271 | - | - | - | - | - |
| Methoxyfenozide | 98.1 | 0.13 | 0.992 | 0.066 | 0.305 | - | - | - | - | - |
| Pirimicarb | 93.3 | 0.28 | 0.996 | 0.160 | 0.304 | 91.3 | 5.79 | 0.993 | 0.257 | 0.338 |

^a Relative standard deviation (%), ^b Lineal range 1.667- 333.4 µg kg⁻¹, ^c Lineal range 0.835-167.1 µg kg⁻¹

3.4 Application to real soil samples

Multiresidue analysis was applied to seventeen real soil samples located in the region of La Rioja using LC-MS. GC-MS was used mainly for the confirmation of those values that might be uncertain because of their high concentration, as in the case of terbuthylazine, fluometuron or acetochlor, Figure 2 includes the distribution of soil samples according to the concentrations of pesticides obtained.

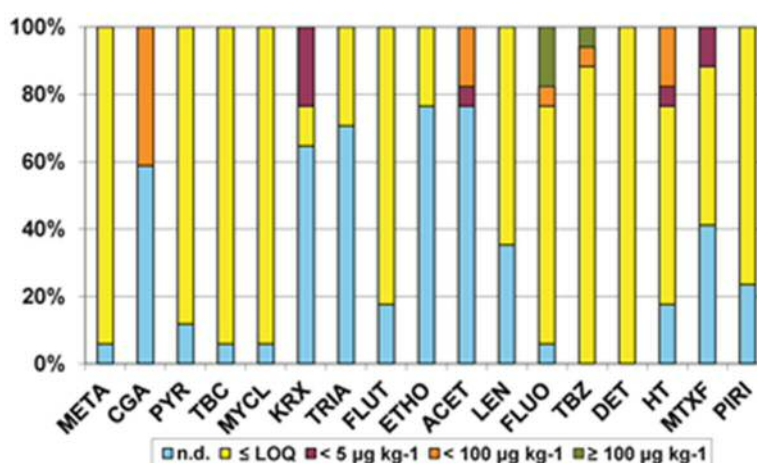


Figure 2. Distribution of real soil samples with not detected concentrations of pesticides or detected concentrations below LOQ and with concentration below $5 \mu\text{g kg}^{-1}$, and $100 \mu\text{g kg}^{-1}$ and over $100 \mu\text{g kg}^{-1}$.

Herbicides were more prominent in all the samples (100% of positive samples for terbuthylazine and DET), although the concentrations in most of the samples were below the LOQ. Only terbuthylazine, and its metabolite HT, and fluometuron recorded concentrations of over $90 \mu\text{g kg}^{-1}$ in some samples. It is important to highlight sample BJ-S12, with concentrations of terbuthylazine, HT and fluometuron of 25.3 , 26.0 and $126 \mu\text{g kg}^{-1}$, respectively, and the sample ALV-S7, with the highest concentrations of terbuthylazine and HT (403 and $39.8 \mu\text{g kg}^{-1}$, respectively), and the samples ALT-S4 and BJ-S11, with the highest concentrations of fluometuron (174 and $142 \mu\text{g kg}^{-1}$). The high concentrations of these herbicides is explained by the greater use of terbuthylazine following the banning of atrazine in 2004 [32], and due to the application of formulations containing both terbuthylazine and fluometuron. This result is consistent with the findings of Herrero-Hernández et al. [24] on the high concentrations reached in surface and ground waters in La Rioja, where terbuthylazine and fluometuron were detected in 67% and 77% of the samples analyzed, reaching peak concentrations of $12.6 \mu\text{g L}^{-1}$ and $18.7 \mu\text{g L}^{-1}$, respectively. The metabolite DET, usually present in waters, is detected only in these soils at concentrations $< \text{LOQ}$, with HT being the metabolite detected at highest concentrations (up to $90.8 \mu\text{g kg}^{-1}$ in BJ-S10). These herbicides have also been detected in other agricultural areas in Spain [25,26,33], and the formation of hydroxylated and dealkylated metabolites by degradation of terbuthylazine in soils has been reported in the literature [34].

The other herbicides studied were detected in waters from this region, but only acetochlor was detected there in concentrations $> 5 \mu\text{g kg}^{-1}$ in some soil samples.

It is remarkable that fungicides were also frequently found in different soils, with a percentage of positive samples of over 80%, except for triadimenol and kresoxim-methyl, with a lower percentage of positive samples (29.4 and 35.3%, respectively), albeit with concentrations > LOQ for kresoxim. The presence of CGA (metabolite of metalaxyl) with 41% of positive samples and concentrations > LOQ is noteworthy. This could be due to a previous application of metalaxyl, present in all the samples, and its degradation route, because the degradation rate of metalaxyl is 42 days [<http://sitem.herts.ac.uk/aeru/footprint/en/index.htm>]. The presence of metalaxyl and myclobutanil have been reported by other authors in surface soil at higher concentrations than those obtained here [15], although in this case the samples were collected only one day after pesticide spraying.

In the case of insecticides, the presence of pirimicarb was detected in a higher percentage of samples than methoxyfenozide, 76.5% of positive samples versus 58.8%, but all these positives were detected below the LOQ. Only methoxyfenozide was detected at higher concentrations in two samples, namely, BJ-S9 and ALT-S4.

It should be noted that high residual amounts were found for hydrophobic compounds such as HT, terbuthylazine, kresoxim-methyl, methoxyfenozide and acetochlor with $\log K_{ow} > 3$ (Table 1). This could be explained by the sorption of these compounds by the OM of soils; indeed, higher percentages of residues were found in soils with a higher OM content (BJ-S12, BJ-S11 and ALT-S4, Table 2). Reports in the literature indicate a significant correlation between the sorption of hydrophobic compounds and soil OM content [35], although it was not possible here to obtain a positive correlation between residual amounts and soil OM content due to the low number of samples with pesticide concentrations > LOQ.

4 CONCLUDING REMARKS

The soil extraction and simultaneous determination of 17 pesticides and the degradation products of the ones most widely used in vineyards was optimized in this work. The extraction solvent was a mixture of methanol:acetone (50:50), with recoveries of over 70% for most of the pesticides although an aqueous solvent was required in the case of HT and CGA. LC-MS and GC-MS methods were optimized and validated with a acceptable linearity ($r^2 \geq 0.987$) within a range of concentrations of 1.667- 333.4 $\mu\text{g kg}^{-1}$ and 0.835-167.1 $\mu\text{g kg}^{-1}$, respectively. However, the determination of all the pesticides selected was only possible by LC-MS and the LODs and LOQs were lower for LC-MS (0.066-0.334 $\mu\text{g kg}^{-1}$ and 0.222 and 0.654 $\mu\text{g kg}^{-1}$) than for GC-MS (0.156-0.387 $\mu\text{g kg}^{-1}$ and 0.263-0.510 $\mu\text{g kg}^{-1}$), which implies more selectivity. The LC-MS method developed was applied to the analysis of 17 vineyard soil samples from the region of La Rioja (Spain). The analysis revealed a strong presence of herbicides in all the samples, with 100% of positive samples for terbuthylazine and DET, and more than 90% for fluometuron, and concentrations recorded of over 90-100 $\mu\text{g kg}^{-1}$ in some samples for these herbicides. The fungicides were also frequently found in the soils, 80% of positive samples, in general, although concentrations were < LOQ, except for kresoxim and CGA. In the case of insecticides, the presence of pirimicarb and methoxyfenozide was detected in > 50% of samples. The results obtained revealed the presence of all the pesticides studied in at least one of the samples analyzed; in all the samples, 68% of the results were positive detections, although only 9% of them were concentrations higher than the LOQ of pesticides.

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Supplementary Material

Table S1. LC-MS and GC-MS chromatographic conditions for determination of pesticides

| Pesticide | LC-MS | | | | GC-MS | | |
|------------------------|-------|----------------------|-----------|---------|----------------|----------------------|----------|
| | M.W. | Quantifier ion (m/z) | V Cone(V) | RT(min) | Monitored ions | | RT (min) |
| | | | | | Target (m/z) | Qualifier ions (m/z) | |
| Metalaxyl | 279.3 | 280.2 | 25 | 17.9 | 45 | 206/132 | 12.6 |
| CGA-62826 | 265.3 | 266.3 | 25 | 5.9 | - | - | - |
| Pyrimethanil | 199.1 | 200.2 | 35 | 20.9 | 198 | 199 | 10.1 |
| Tebuconazole | 307.8 | 308.2 | 25 | 23.2 | 125 | 250/70 | 23.7 |
| Myclobutanil | 288.8 | 289.1 | 25 | 20.5 | 179 | 82/150 | 20.8 |
| Kresoxim-methyl | 313.5 | 314.4 | 20 | 22.8 | 116 | 131/206 | 21.1 |
| Triadimenol | 295.7 | 296.2 | 15 | 20.7 | 112 | 128/168 | 15.8 |
| Flutriafol | 301.2 | 303.2 | 20 | 16.7 | 123 | 164/219 | 19.8 |
| Ethofumesate | 286.3 | 287.2 | 20 | 20.1 | 207 | 161/137 | 13.6 |
| Acetochlor | 269.7 | 270.2 | 15 | 21.8 | 59 | 146/162 | 10.9 |
| Lenacil | 234.2 | 235.2 | 15 | 17.4 | 153 | 53/154 | 25.5 |
| Fluometuron | 232.2 | 233.2 | 25 | 18.0 | 174 | 219/187 | 8.3 |
| Terbuthylazine | 229.7 | 230.2 | 25 | 20.2 | 214 | 43/173 | 9.5 |
| Desethylterbuthylazine | 201.6 | 202.2 | 20 | 15.9 | 186 | 188/83 | 8.6 |
| Hydroxyterbuthylazine | 211.3 | 212.2 | 25 | 15.6 | - | - | - |
| Methoxyfenozide | 368.4 | 369.3 | 20 | 20.5 | - | - | - |
| Pirimicarb | 238.3 | 239.2 | 20 | 18.4 | 16.6 | 72/238 | 11.4 |

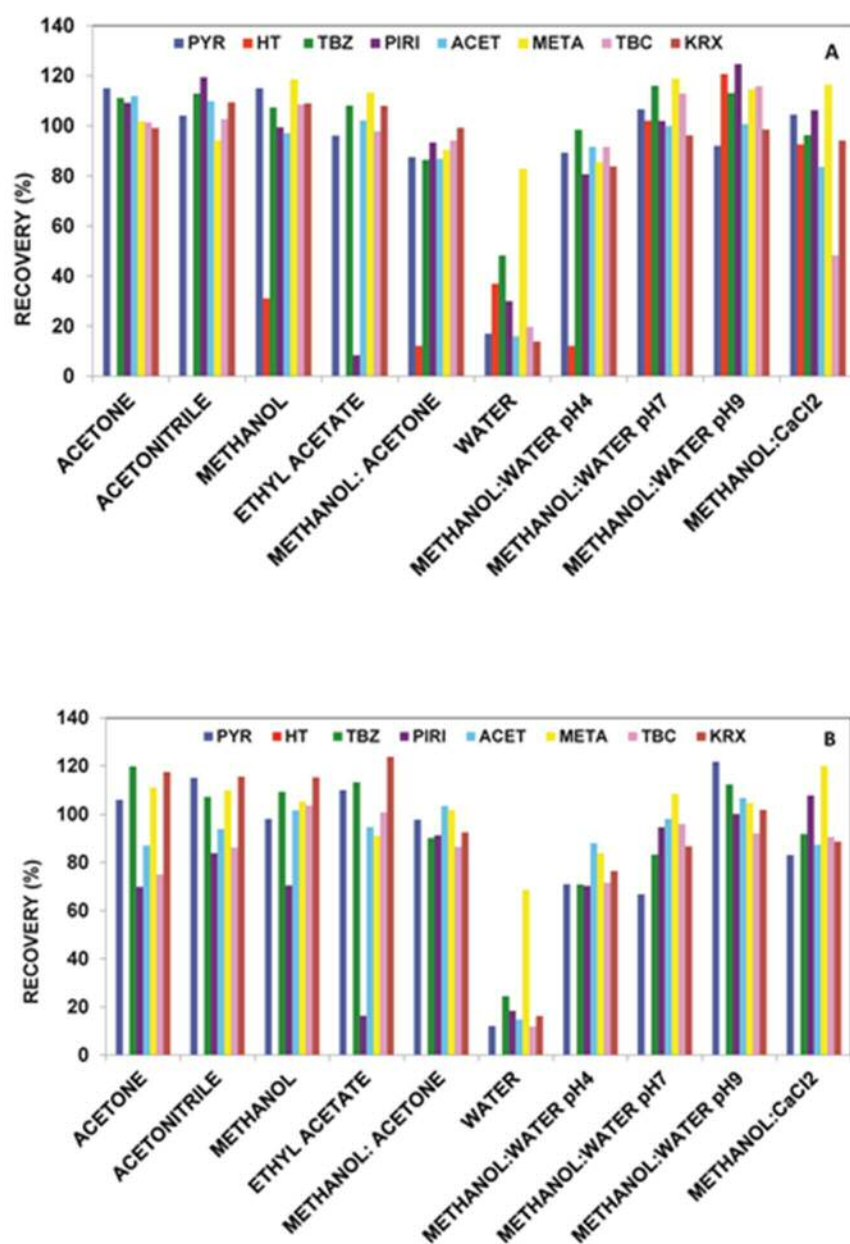


Figure S1. Recovery (%) obtained for some pesticides from soil spiked at 0.1 mg kg^{-1} extracted with different solvents tested and determined by LC-MS (A) and GC-MS (B).

Application of a biosorbent to soil: a potential method for controlling water pollution by pesticides

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Abstract

Different strategies are now being optimized to prevent water from agricultural areas being contaminated by pesticides. The aim of this work was to optimize the adsorption of non-polar (tebuconazole, triadimenol) and polar (cymoxanil, pirimicarb) pesticides by soils after applying the biosorbent spent mushroom substrate (SMS) at different rates. The adsorption isotherms of pesticides by three soils and SMS-amended soils were obtained and the adsorption constants were calculated. The distribution coefficients (K_d) increased 1.40-23.1 times (tebuconazole), 1.08-23.7 times (triadimenol), 1.31-42.1 times (cymoxanil) and 0.55-23.8 times (pirimicarb) for soils amended with biosorbent at rates between 2% and 75%. Increasing the SMS rates led to a constant increase in adsorption efficiency for non-polar pesticides but not for polar pesticides, due to the increase in the OC content of soils as indicated by K_{OC} values. The OC content of SMS-amended soils accounted for more than 90% of the adsorption variability of non-polar pesticides, but it accounted for only 56.3% for polar pesticides. The estimated adsorption of SMS-amended soils determined from the individual adsorption of soils and SMS was more consistent with real experimental values for non-polar pesticides than for polar pesticides. The results revealed the use of SMS as a tool to optimize pesticide adsorption by soils in dealing with specific contamination problems involving these compounds.

Keywords: Biosorbent, spent mushroom substrate, immobilization, soil, non-polar pesticides, polar pesticides, water pollution

1 INTRODUCTION

The waste material called spent mushroom substrate (SMS) is the soil-like material remaining after a crop of mushrooms has been harvested. The production of 1 kg of mushroom produces 5 kg of SMS. China has the largest production in the world (20 Mt) (Phan and Sabaratnam 2012). In addition, the USA, the Netherlands, France, Ireland, Poland and Spain are currently major producers (FAOSTAT 2015).

In Spain, mushroom production represented about 7.63% of the entire European Union (EU) production in 2012 (FAOSTAT 2015). In the region of La Rioja increasing quantities of SMS are being produced (about 170 000 tonnes of SMS per year) (MAGRAMA 2015). It has been reported that the SMS addition to a semiarid vineyard soil in this region could be a promising strategy for the sustainable recycling of this waste avoiding its ultimate disposal in landfills. The SMS addition increases the organic

matter (OM) of these soils with low OM content, which have beneficial effects on soil quality, especially on soil biochemical properties and microbiological parameters (Medina et al. 2012; Peregrina et al. 2012).

Besides these potential benefits, SMS has a major ability to adsorb pesticides from water (Marín-Benito et al. 2012a). The role it plays in controlling the behaviour of the pesticides applied, or which enter the soil along with the SMS, has been investigated. The OM applied with the SMS might influence the sorption capacity of pesticides by soils and modify their concentration in the soil solution, decreasing their toxic effects (Gómez et al. 2014). Studies have focused especially on the influence on the adsorption and degradation of certain fungicides frequently used in vineyards depending on the nature of the SMS, as determined by its degree of composting, or the length of time the soil has been incubated with the waste (Marín-Benito et al. 2012b). The results obtained following the application of SMS to the soil have revealed a decrease in adsorption over the waste's incubation time with the soil, and the influence of SMS organic carbon (OC) and dissolved organic carbon (DOC) on the adsorption and dissipation processes of relatively hydrophobic fungicides. The more hydrophobic pesticides recorded a significant increase in adsorption by the SMS-amended soil, even at a dose of 2%, although the presence of this waste was not so efficient for the adsorption of less hydrophobic pesticides.

Nevertheless, in view of the current interest in avoiding the contamination of groundwater due to the leaching of the pesticides that reach the soil, it was considered expedient to discover whether the application to the soil of different SMS rates may allow optimising the adsorption of different kinds of pesticides depending on the need to address specific environmental issues. Pesticides are organic compounds that are widely used in high amounts in order to increase the quality and yield of crops. Their presence in waters in concentrations higher than those allowed by legislation on drinking water (Directive No. 2008/105/EC, amending the Water Framework Directive 2000/60/EC) is becoming more common (Herrero-Hernández et al. 2013; Masiá et al. 2015). These concentrations in the water may come from diffuse contamination due to the agricultural use of these compounds, although in many cases they have been attributed to point soil contamination due to the improper disposal of empty containers or the washing of equipment after the pesticide's application (Fait et al. 2007).

Good agro-environmental practices have been proposed to avoid diffuse contamination, while biobeds or biomix systems have been described for avoiding point contaminations (Castillo et al. 2008). In both cases, the application and/or use of wastes from different origins with a high percentage of OM in their composition are of great interest for avoiding environmental pollution in the most cost-effective ways possible (Beesley et al. 2011). The adsorption of pesticides by the OM of the residues has an effect on their degradation rate and bioavailability, and determines the concentration of pesticides in the soil solution and how they are transported throughout the medium (Dolaptsoglou et al. 2007; Rodríguez-Salgado et al. 2014). Accordingly, adsorption studies are a good method for measuring the adsorbent capacity of the modified soil by the addition of wastes from different origins. After pesticides have been sequestered in the amended soil, they could remain in the soil as chemically adsorbed, physically trapped, or a combination of both processes (Zhang et al. 2011; Ahmad et al. 2014), thus decreasing the likelihood of being leached (Fenoll et al. 2011; Kodešová et al. 2011).

The fungicides tebuconazole, triadimenol and cymoxanil are widely used in vineyards to combat powdery mildew fungi, and pirimicarb is an insecticide used to control aphids. These pesticides could

reach the soil after their application to plants, and we have detected them in groundwaters in the region of La Rioja (Herrero-Hernández et al., 2013).

The aim of this study is to evaluate the capacity of a SMS-amended soil to adsorb two non-polar pesticides (tebuconazole and triadimenol) and two polar pesticides (cymoxanil and pirimicarb) with different hydrophobic character and water solubility, and determine the effect that different rates of biosorbent have on the immobilization of these pesticides. The adsorption of these compounds by three soils amended with SMS at different rates was carried out in order to discover the following: i) the effect of SMS when applied to soil as an organic amendment (lower rates 2-10%) on the adsorption of pesticides used in agricultural practices, and ii) the effect of SMS when applied to soil as a barrier (higher rates, 25-75%) on the adsorption/immobilization of pesticides to prevent the pollution of groundwater.

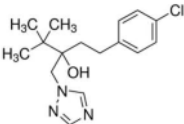
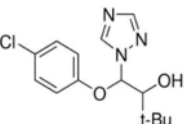
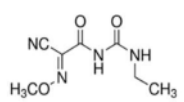
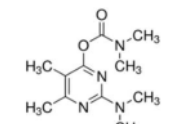
2 MATERIALS AND METHODS

2.1 Chemicals

Pesticides studied were three fungicides (tebuconazole, cymoxanil and triadimenol) and one insecticide (pirimicarb). Table 1 shows their characteristics (PPDB 2015). Two pesticides selected are classified as non-polar and immobile (tebuconazole) or mobile (triadimenol) and two more pesticides are classified as polar and immobile (pirimicarb) or mobile (cymoxanil) according to the classification of non-polar when the log Kow value is > 3.0 and as mobile when the log Koc is < 2.5 (Delle Site 2001).

Tebuconazole and cymoxanil were used ^{14}C -labelled with specific activity and purity of 4.72 MBq mg^{-1} and 98.1 % for ^{14}C -tebuconazol and 10.08 MBq mg^{-1} and 97.79 % for ^{14}C -cymoxanil. Both pesticides were supplied by IZOTOP (Hungary). Unlabelled pesticides (purity $> 98\%$) were supplied by Sigma-Aldrich Química SA (Madrid, Spain).

Table 1. Chemical structure and physicochemical properties of pesticides studied^a

| Common name IUPAC name | Chemical structure | WS (mg L^{-1}) | Log Kow | Koc(mL g^{-1}) | DT ₅₀ (days ⁻¹) | GUS index |
|--|---|------------------------------|---------|---------------------------|---|--------------|
| Tebuconazole (<i>RS</i>)-1-p-chlorophenyl-4,4-dimethyl-3-(1 <i>H</i> -1,2,4-triazol-1-ylmethyl)pentan-3-ol |  | 36 | 3.7 | 769 | 63-365 | 2.0 |
| Triadimenol (1 <i>RS</i> ,2 <i>RS</i> ;1 <i>RS</i> ,2 <i>SR</i>)-1-(4-chlorophenoxy)-3,3-dimethyl-1-(1 <i>H</i> -1,2,4-triazol-1-yl)butan-2-ol |  | 72 | 3.18 | 273 | 64.9-250 | 3.75 |
| Cymoxanil 1-[(<i>EZ</i>)-2-cyano-2-methoxyiminoacetyl]-3-ethylurea |  | 780 | 0.67 | 43.6 | 0.7-3.5 | -0.37 |
| Pirimicarb 2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate |  | 3100 | 1.7 | 388 | 86 | 2.73 |

^a From PPDB, Pesticide Properties DataBase (2015) <http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm>. WS solubility in water at 20 °C, Kow octanol/water partition coefficient at pH 7 and 20°C, Koc sorption coefficient normalized to organic carbon content.

2.2 Biosorbent and Soils

Biosorbent spent mushroom substrate (SMS) from *Agaricus bisporus* cultivation was supplied by Sustratos de La Rioja S.L. (Pradejón, Spain). Its composition was described by Marín-Benito et al. (2012b) and their physicochemical characteristics determined as described in this previous work are: pH was 6.97, ash content was 33.6%, OC content was 24.5%, DOC was 1.91% and moisture content was 64.5%.

Soil samples were collected from the surface horizon (0-30 cm in three vineyards in La Rioja region (Spain) located in Alcanadre (ALC) (42°24'20"N, 2°7'6"W) in La Rioja Baja, Sajazarra (SJZ) (42°35'18"N, 2°57'41"W) and Yécora (YEC) (42°32'47"N, 3°5'48"W) both in La Rioja Alta. The soils were air-dried and sieved (<2 mm) to determine their characteristics using standard analytical methods (MAPA 1986). Soils selected had low OM contents and different clay contents. Their textures were classified as sandy loam, sandy clay loam and clay loam, respectively.

Soils were amended with the biosorbent by uniformly mixing of soil with appropriate amounts of SMS sieved (<2mm) to obtain rates of 2%, 5%, 10%, 25%, 50% and 75% (w/w), on a dry weight basis. The initial moisture content of all soils was adjusted to 40% of their maximum water holding capacity. Adsorption assays were conducted after some days of SMS-soil incubation to equilibrate. Characteristics of unamended and amended soils are included in Table 2.

Table 2. Characteristics of unamended and amended soils

| Soil | pH | OC % | DOC % | OM % | N % | C/N | CO ₃ ²⁻ % | Sand % | Silt % | Clay % | Clay mineralogy ^a |
|-----------|------|---------|----------|---------|--------|------|------------------------------------|-----------|-----------|-----------|---------------------------------|
| ALC | 7.49 | 0.89 | <0.01 | 1.53 | 0.10 | 9.10 | 24.1 | 79.6 | 9.0 | 11.4 | I, K, V |
| ALC+SMS2 | 7.39 | 1.22 | 0.056 | | | | | | | | |
| ALC+SMS5 | 7.26 | 2.22 | 0.062 | | | | | | | | |
| ALC+SMS10 | 7.22 | 4.00 | 0.277 | | | | | | | | |
| ALC+SMS25 | 7.15 | 7.11 | 0.342 | | | | | | | | |
| ALC+SMS50 | 7.10 | 15.6 | 0.446 | | | | | | | | |
| ALC+SMS75 | 7.06 | 18.9 | 1.063 | | | | | | | | |
| SJZ | 7.52 | 0.67 | <0.01 | 1.15 | 0.06 | 12.3 | 50.9 | 67.0 | 11.9 | 21.1 | I, K |
| SJZ+SMS2 | 7.40 | 0.97 | 0.042 | | | | | | | | |
| SJZ+SMS5 | 7.26 | 1.73 | 0.062 | | | | | | | | |
| SJZ+SMS10 | 7.25 | 3.56 | 0.199 | | | | | | | | |
| SJZ+SMS25 | 7.21 | 6.25 | 0.399 | | | | | | | | |
| SJZ+SMS50 | 7.19 | 16.3 | 0.439 | | | | | | | | |
| SJZ+SMS75 | 7.09 | 17.6 | 1.211 | | | | | | | | |
| YEC | 7.84 | 1.00 | <0.01 | 1.72 | 0.09 | 10.4 | 12.9 | 34.6 | 29.7 | 35.7 | I, K, M |
| YEC+SMS2 | 7.70 | 1.78 | 0.038 | | | | | | | | |
| YEC+SMS5 | 7.46 | 2.22 | 0.047 | | | | | | | | |
| YEC+SMS10 | 7.56 | 4.89 | 0.169 | | | | | | | | |
| YEC+SMS25 | 7.36 | 9.33 | 0.454 | | | | | | | | |
| YEC+SMS50 | 7.29 | 12.2 | 0.442 | | | | | | | | |
| YEC+SMS75 | 7.59 | 19.3 | 0.840 | | | | | | | | |

^aIllite (I), Kaolinite (K), Montmorillonite (M), Vermiculite (V).

2.3 Adsorption experiments

Adsorption isotherms of pesticides by unamended and amended soils were obtained using the batch equilibrium technique. Adsorption of tebuconazole and cymoxanil was carried out by ALC, SJZ and YEC soils and adsorption of triadimenol and pirimicarb was carried out by ALC and SJZ soils. Duplicate soil samples (1g) were equilibrated with 10 mL of a Milli-Q ultrapure water solution of each pesticide at concentrations of 1, 5, 10, 15, 20 and 25 $\mu\text{g mL}^{-1}$ for non-polar pesticides and 1, 5, 10, 15, 20, 25, 50 and 100 $\mu\text{g mL}^{-1}$ for polar pesticides, according to the solubility range of compounds. The soil:solution ratio was adapted to obtain a suitable adsorption according to the OECD guideline (OECD/OCDE, 2000). An activity of 100 Bq mL^{-1} was used for ^{14}C -tebuconazole and ^{14}C -cymoxanil. The suspensions were shaken at $20\pm 2^\circ\text{C}$ for 24 h in a thermostated chamber, with intermittent shaking for 2 h at 3 h intervals. Preliminary experiments revealed that contact for 24 h was long enough for attaining equilibrium. The suspensions were subsequently centrifuged at 5045 g for 15 min, and the equilibrium concentration of each pesticide was determined. The amount of pesticide adsorbed was calculated as the difference between that initially present in solution and that remaining after equilibration with the soil. Blanks (soils without pesticide or standard solutions without soil) were prepared and managed in a similar way to samples and used as controls. Calculations were based on the assumption that no degradation of fungicides occurred during sorption studies.

2.4 Pesticide analysis

The equilibrium concentrations of ^{14}C -tebuconazole and ^{14}C -cymoxanil in the supernatant were determined by liquid scintillation counting on a Beckman LS6500 liquid scintillation counter (Beckman Instrument Inc., Fullerton, CA). The radioactivity of the equilibrium solution was measured in disintegrations per minute (dpm) as indicated in Marín-Benito et al. (2012b).

Pirimicarb and triadimenol were determined by HPLC-MS (Waters Association, Milford, MA), using a Phenomenex Luna (3 μ C18, 150 x 4.60 mm) column. The mobile phase was in both cases 80:20 (v:v) acetonitrile:water (0.1% formic acid). The flow rate was 0.3 mL min^{-1} , and the sample injection volume was 20 μL . Calibration curves were generated between 0.1-100 $\mu\text{g mL}^{-1}$ (pirimicarb) and between 0.1-25 $\mu\text{g mL}^{-1}$ (triadimenol) concentrations of standards in solutions of adsorbent extracts to counteract any possible matrix effect. Retention times for pirimicarb and triadimenol were 4.5 and 5.1 min, respectively. Quantitative analysis was performed using the peak area of each compound obtained from the total ion chromatogram in SIM mode. The positive molecular ion (m/z) was 239.29 for pirimicarb and 296.76 for triadimenol. The limits of detection (LOD) were 0.014 and 0.011 $\mu\text{g L}^{-1}$ and the limits of quantification (LOQ) were 0.039 and 0.036 $\mu\text{g L}^{-1}$ for pirimicarb and triadimenol, respectively.

2.5 Data analysis

Pesticide adsorption data were fitted to the Freundlich equation $C_s = K_f C_e^{n_f}$, where C_s ($\mu\text{g g}^{-1}$) is the amount of adsorbed pesticide; C_e ($\mu\text{g mL}^{-1}$) is the equilibrium concentration of pesticide solution, and K_f ($\mu\text{g}^{1-n_f} \text{g}^{-1} \text{mL}^{n_f}$) and n_f are the Freundlich adsorption and non-linearity coefficients, respectively. Distribution coefficients, K_d (mL g^{-1}), were determined from the relationship between C_s and C_e , and K_d

values normalised to 100% OC (Koc) were calculated. Standard deviation (SD) was used to indicate variability in the adsorption coefficients among replicates. Simple and multiple linear regression models were used to relate adsorption to the soil characteristics. IBM SPSS (version 22; USA) statistical software was used.

3 RESULTS AND DISCUSSION

3.1 Effect of different rates of SMS on the adsorption of non-polar pesticides

Figure 1 includes the adsorption isotherms of tebuconazole and triadimenol for by unamended soils, SMS, and soils amended with rates of SMS in the 2-75% range. All the isotherms fitted the Freundlich equation, with r values ≥ 0.99 and ≥ 0.95 , respectively. K_f and n_f parameters were determined from the linear form of this equation, and they are included in Table 3. The adsorption isotherms of tebuconazole for unamended soils were non-linear (L-type), although an increasing linearity was seen for the isotherms of fungicide for soils amended at different rates. These isotherms indicated that adsorption decreases at high concentrations of pesticide in solution. Similar non-linear shapes were obtained for the adsorption isotherms of triadimenol for unamended and amended soils, although the increase in linearity was less significant. Adsorption and partitioning mechanisms may explain the retention of these non-polar pesticides as a function of the rates of SMS in the soils. These adsorption mechanisms have been reported for the adsorption of other non-polar pesticides or organic contaminants by amended soils with different organic materials (Eibisch et al. 2015).

K_f values for the adsorption of tebuconazole and triadimenol by unamended soils were in the 2.55-5.03 and 1.69-1.81 range, respectively, and 53.8 and 57.9, respectively, for SMS. OC increased after the SMS amendment of soils and enhanced the adsorption of both pesticides by SMS-amended soils, with the increased soil adsorption of tebuconazole being higher than that of triadimenol (Table 3). Significant correlation coefficients were found between K_f values and OC contents of soils (r ranged between 0.978 and 0.992 for tebuconazole and between 0.953 and 0.978 for triadimenol ($p < 0.01$)). DOC also increased after the SMS amendment of soils, and a positive significant correlation was recorded between K_f and DOC of soils ($r = 0.903$ - 0.918 for tebuconazole ($p < 0.05$) and $r = 0.994$ - 0.977 for triadimenol ($p < 0.01$)). A significant correlation coefficient was found between OC and DOC, although DOC did not decrease the adsorption of these pesticides by amended soils, as observed for other organic residues and/or pesticides. Reports have indicated increases or decreases in the adsorption of pesticides by soils because of the effect of DOC (Andrades et al. 2004; Rodríguez-Cruz et al. 2012). In this case, soluble organic compounds could be adsorbed by soil components, giving rise to the formation of new hydrophobic surfaces that would increase pesticide adsorption.

The K_d distribution coefficients were determined at an equilibrium concentration of $5 \mu\text{g mL}^{-1}$ to compare the adsorption capacity of pesticides by soils amended with different rates of SMS (2-75%), because the n_f values of the adsorption isotherms were different to 1. K_{d5} varied between 1.99 and 3.91 (tebuconazole) and between 1.36 and 1.50 (triadimenol) for unamended soils, and these values increased after amendment (Table 3). Increasing the SMS rates led to a constant increase in the adsorption efficiency for both pesticides. The higher adsorption of pesticides by ALC, SJZ and YEC amended soils was somewhat similar, and apparently depended only on the total OC content of the SMS-treated soils. In

fact, the relative standard deviation (RSD) of K_d values normalised to OC content (K_{oc}) for amended soils varied between 19.7 and 28.7% for tebuconazole, and between 23.9 and 25.1% for triadimenol. This variability of K_{oc} values was lower than that usually found between the adsorption coefficients of pesticides for soils normalized to soil OC content (Papiernik et al. 2006), and ascribed to the different nature of soil OC.

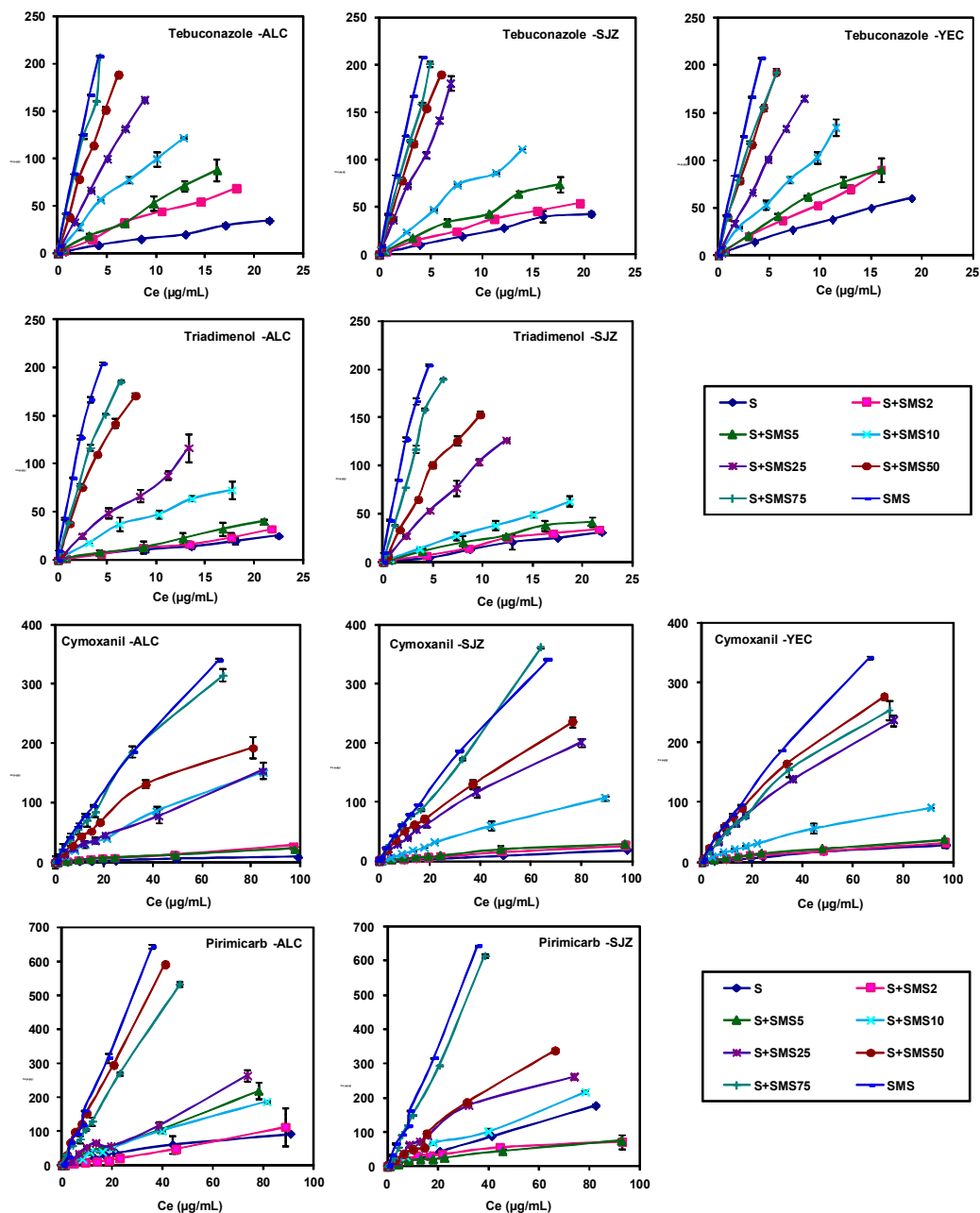


Fig. 1. Adsorption isotherms of non-polar and polar pesticides by unamended soils, SMS and amended soils at different SMS-rates. Error bars represent the standard deviation of the C_s mean value ($n = 2$)

Table 3. Freundlich adsorption constants and distribution coefficients of non-polar pesticides by soils amended at different SMS rates

| Soil | Tebuconazole | | | K_{OC} | Triadimenol | | | |
|-----------|-----------------|-----------------|-----------------|----------|-----------------|-----------------|-----------------|----------|
| | $K_f \pm SD^a$ | $nf \pm SD$ | $Kd5^b \pm SD$ | | $K_f \pm SD$ | $nf \pm SD$ | $Kd5 \pm SD$ | K_{OC} |
| ALC | 2.55 \pm 0.26 | 0.84 \pm 0.03 | 1.99 \pm 0.11 | 224 | 1.81 \pm 0.82 | 0.83 \pm 0.11 | 1.36 \pm 0.11 | 153 |
| ALC+SMS2 | 4.94 \pm 0.01 | 0.92 \pm 0.03 | 4.34 \pm 0.39 | 356 | 1.63 \pm 1.01 | 0.94 \pm 0.18 | 1.46 \pm 0.46 | 120 |
| ALC+SMS5 | 6.76 \pm 0.28 | 0.90 \pm 0.02 | 5.73 \pm 1.54 | 258 | 2.41 \pm 0.94 | 0.88 \pm 0.14 | 1.99 \pm 0.80 | 89.7 |
| ALC+SMS10 | 13.5 \pm 0.93 | 0.89 \pm 0.01 | 11.2 \pm 3.21 | 281 | 6.45 \pm 1.02 | 0.87 \pm 0.02 | 5.26 \pm 0.05 | 132 |
| ALC+SMS25 | 20.0 \pm 0.03 | 0.98 \pm 0.00 | 19.8 \pm 0.49 | 271 | 9.11 \pm 1.36 | 0.97 \pm 0.01 | 8.74 \pm 1.21 | 123 |
| ALC+SMS50 | 34.6 \pm 0.64 | 0.94 \pm 0.02 | 31.5 \pm 2.22 | 202 | 25.4 \pm 0.32 | 0.93 \pm 0.01 | 22.6 \pm 2.36 | 145 |
| ALC+SMS75 | 48.9 \pm 1.77 | 0.96 \pm 0.02 | 45.9 \pm 2.57 | 243 | 34.3 \pm 3.55 | 0.96 \pm 0.04 | 32.3 \pm 1.62 | 171 |
| SJZ | 3.06 \pm 0.06 | 0.89 \pm 0.01 | 2.57 \pm 0.10 | 383 | 1.69 \pm 0.43 | 0.92 \pm 0.05 | 1.50 \pm 0.25 | 224 |
| SJZ+SMS2 | 4.71 \pm 0.67 | 0.83 \pm 0.07 | 3.60 \pm 0.55 | 371 | 2.32 \pm 0.78 | 0.88 \pm 0.11 | 1.75 \pm 0.41 | 180 |
| SJZ+SMS5 | 6.24 \pm 0.04 | 0.87 \pm 0.01 | 5.03 \pm 1.36 | 290 | 3.08 \pm 0.94 | 0.88 \pm 0.11 | 2.54 \pm 0.35 | 147 |
| SJZ+SMS10 | 9.16 \pm 0.78 | 0.96 \pm 0.02 | 8.61 \pm 0.79 | 242 | 4.71 \pm 0.31 | 0.87 \pm 0.02 | 3.88 \pm 0.48 | 109 |
| SJZ+SMS25 | 26.9 \pm 1.59 | 0.95 \pm 0.03 | 24.9 \pm 5.77 | 398 | 10.9 \pm 0.59 | 0.99 \pm 0.00 | 10.6 \pm 1.02 | 169 |
| SJZ+SMS50 | 36.6 \pm 0.38 | 0.94 \pm 0.00 | 33.1 \pm 0.29 | 203 | 18.9 \pm 0.83 | 0.98 \pm 0.05 | 18.1 \pm 1.96 | 111 |
| SJZ+SMS75 | 42.0 \pm 0.05 | 0.97 \pm 0.00 | 39.9 \pm 0.41 | 226 | 35.6 \pm 3.01 | 0.90 \pm 0.09 | 34.4 \pm 2.47 | 195 |
| YEC | 5.03 \pm 0.02 | 0.84 \pm 0.02 | 3.91 \pm 0.08 | 391 | | | | |
| YEC+SMS2 | 7.10 \pm 0.05 | 0.90 \pm 0.00 | 6.07 \pm 0.30 | 341 | | | | |
| YEC+SMS5 | 8.17 \pm 0.23 | 0.90 \pm 0.04 | 6.93 \pm 0.72 | 312 | | | | |
| YEC+SMS10 | 13.9 \pm 0.14 | 0.92 \pm 0.02 | 11.9 \pm 0.20 | 245 | | | | |
| YEC+SMS25 | 21.6 \pm 0.98 | 0.96 \pm 0.06 | 20.2 \pm 1.42 | 216 | | | | |
| YEC+SMS50 | 38.2 \pm 1.46 | 0.93 \pm 0.01 | 35.4 \pm 0.28 | 290 | | | | |
| YEC+SMS75 | 40.5 \pm 0.34 | 0.95 \pm 0.02 | 36.3 \pm 0.68 | 188 | | | | |
| SMS | 53.8 \pm 1.32 | 0.95 \pm 0.01 | 49.9 \pm 4.62 | 203 | 57.9 \pm 0.58 | 0.90 \pm 0.03 | 48.9 \pm 7.90 | 199 |

^a SD, standard deviation (n = 2). ^b Distribution coefficient for a equilibrium concentration of 5 $\mu\text{g mL}^{-1}$

However, it should be noted that the efficiency of SMS applied to increase adsorption was relative to the adsorption capacity of pesticides by the unamended soil. Figure 2 includes the increases in $Kd5$ after amendment relative to unamended soil (i.e., $Kd5$ amended soil/ $Kd5$ unamended soil) for tebuconazole and triadimenol in the soils studied. The minor efficiency of SMS for the retention of these pesticides was seen in YEC soil for tebuconazole and in SJZ soil for triadimenol due to the greater adsorption of these pesticides by these unamended soils. The higher clay content of both soils or the presence of montmorillonite in YEC soil -a clay mineral with a greater adsorption capacity (Sánchez-Martín et al. 2006; Báez et al. 2015) may explain the increased adsorption of pesticides by these soils and the lower global adsorption by the soil+SMS.

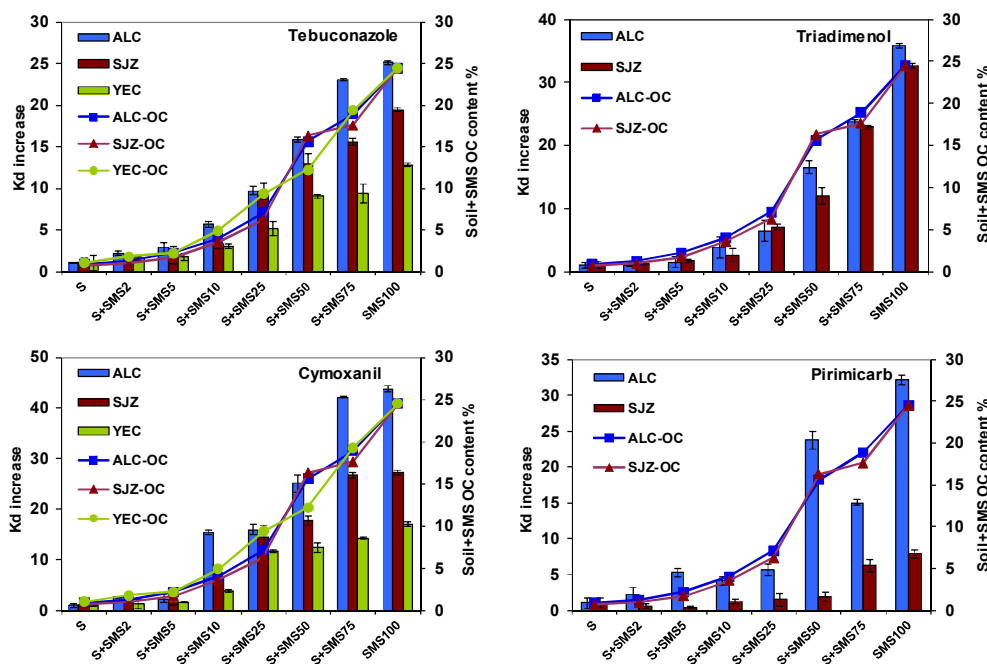


Fig. 2. Increases in adsorption distribution coefficients (Kd) of non-polar and polar pesticides by amended soils at different SMS-rates and organic carbon (OC) of amended soils. Error bars represent the standard deviation of the mean value (n=2).

The results indicated that a further increase in Kd5 by up to three-to-five times may be achieved for both compounds when an amendment rate $\leq 10\%$ was applied to the soils, with the highest increase for the ALC soil with the lowest adsorption capacity. This rate corresponds to an application of $\approx 25\text{-}30 \text{ t C ha}^{-1}$, which could equal an agronomic rate of SMS when this organic waste was used as an amendment. In this case, the adsorption of pesticides by the amendment applied could avoid the rapid leaching of these compounds or the diffuse contamination of waters by the pesticides when they reach the soils. However, a higher application of this organic residue could be used when the immobilization of pesticides was the objective for the prevention of water contamination from a point pollution source due to the management of these compounds. Increases in Kd of up to more than twenty times could then be achieved by applying an appropriate SMS rate to the amended soils.

The effect of SMS on the retention of tebuconazole and triadimenol was corroborated statistically as being due mainly to the OC content from SMS. A highly significant correlation coefficient was obtained between Kd5 coefficients for tebuconazole and triadimenol jointly considered with the soil OC content ($p < 0.001$). On the basis of the determination coefficient r^2 , the OC would account for 90.8% of the variance in adsorption according to the equation: $Kd5 = 1.039 (\pm 1.137) + 1.923 (\pm 0.103) \text{ OC}$. The results were also subjected to a multiple linear regression analysis by combining two or more variables to determine the relative importance of soil parameters and/or pesticide parameters when they vary simultaneously. The inclusion of DOC and soil clay content in the multiple correlation model did not increase the variance in adsorption ($R^2 = 89.8$). However, the inclusion of the K_{ow} of pesticides according to the positive correlation generally reported between the hydrophobicity of organic pollutants and adsorption (Nam et al. 2014) revealed a significant increase in the variance of adsorption, with the

relationship between K_{d5} adsorption constants and the OC content and K_{ow} following the equation: $K_{d5} = -39.65 (\pm 8.419) + 1.931 (\pm 0.0081) OC + 11.65 (\pm 2.396) K_{ow}$. The determination coefficient $R^2 = 94.6\%$ accounts for a higher percentage of K_{d5} variability than that explained when only the OC content is considered as the responsible variable. The equation obtained could be used to predict the adsorption of a non-polar pesticide from the increase in OC content by applying different rates of SMS to soil as a function of the purpose to be achieved.

3.2 Effect of different rates of SMS on the adsorption of polar pesticides by soils

The adsorption isotherms of cymoxanil and pirimicarb for unamended soils, SMS and soils amended with doses of SMS in the 2-75% range are included in Fig. 1. All the isotherms fitted the Freundlich equation with r values ≥ 0.98 and ≥ 0.96 , respectively. K_f and n_f parameters were determined from the linear form of this equation (Table 4). All the adsorption isotherms of cymoxanil for unamended and amended soils were non-linear (L-type), with n_f values in general < 0.90 . No changes in linearity were observed for the isotherms of cymoxanil adsorbed by soils amended at different rates, as indicated for non-polar pesticides. Similar non-linear shapes were obtained for the adsorption isotherms of pirimicarb for unamended and amended soils, although the isotherms of this compound were S- or L-type, being unrelated to the SMS rate applied. Only adsorption mechanisms can explain the retention of these pesticides by SMS-soils, as reported for polar pesticides in general (Wang et al. 2011).

The K_f values for the adsorption of cymoxanil and pirimicarb were in the 0.29-0.58 and 0.44-0.78 ranges, respectively, for unamended soils, and 8.74 and 13.8 respectively, for SMS. The adsorption coefficients by soils and SMS are up to ten times and up to five times lower than those obtained for non-polar pesticides. The increase in soil OC content after SMS amendment enhanced the adsorption of both polar pesticides by amended soils (Table 4), and a significant correlation coefficient was also found between the K_f values and OC content of soils, although these r values were lower for polar pesticides than for non-polar pesticides. A positive correlation was also obtained between K_f and DOM for both pesticides although it was not always significant ($r = 0.793-0.914$ ($p < 0.1 - 0.05$) for cymoxanil and $r = 0.683-0.936$ for pirimicarb ($p > 0.10 - < 0.01$)). There could be interactions between DOC molecules from amendments and mineral soil surfaces competing with polar pesticide molecules for the same mineral adsorption sites.

To compare the sorption capacity of pesticides by soils amended with different rates of SMS, the K_d distribution coefficients were determined at an equilibrium concentration, because n_f values were different to 1. An equilibrium concentration of $40 \mu\text{g mL}^{-1}$ was selected from the wide range of concentrations considered for the adsorption isotherms. K_{d40} varied for unamended soils between 0.12 and 0.33 for cymoxanil and between 0.62 and 2.54 for pirimicarb (Table 4) and these values increased after amendment compared to the unamended soil. The effect of SMS on the retention of cymoxanil and pirimicarb increased with the OC content provided by the SMS, as indicated by non-polar pesticides. However, it was noted that the RSD of K_{d40} values normalised to OC content for amended soils fell within a wide range 23-44% (cymoxanil) and 40-94% (pirimicarb), indicating that other soil properties could also affect the adsorption of these pesticides by amended soils.

Table 4. Freundlich adsorption constants and distribution coefficients of polar pesticides by soils amended at different SMS rates

| Soil | Cymoxanil | | | Pirimicarb | | | | |
|-----------|----------------------|-------------|------------------------|-----------------|-------------|-------------|-------------|-----------------|
| | Kf ± SD ^a | nf ± SD | Kd40 ^b ± SD | K _{OC} | Kf ± SD | nf ± SD | Kd40 ± SD | K _{OC} |
| ALC | 0.46 ± 0.00 | 0.78 ± 0.02 | 0.12 ± 0.00 | 14.5 | 0.44 ± 0.16 | 0.87 ± 0.03 | 0.62 ± 0.82 | 69.9 |
| ALC+SMS2 | 0.82 ± 0.12 | 0.75 ± 0.01 | 0.33 ± 0.02 | 27.0 | 2.29 ± 0.10 | 1.11 ± 0.03 | 1.41 ± 0.01 | 116 |
| ALC+SMS5 | 0.99 ± 0.10 | 0.61 ± 0.03 | 0.28 ± 0.01 | 13.0 | 2.57 ± 0.61 | 1.06 ± 0.09 | 3.26 ± 0.37 | 147 |
| ALC+SMS10 | 3.65 ± 0.14 | 0.83 ± 0.01 | 1.98 ± 0.30 | 49.6 | 3.46 ± 0.10 | 0.92 ± 0.03 | 2.56 ± 0.22 | 64.1 |
| ALC+SMS25 | 4.39 ± 0.43 | 0.79 ± 0.03 | 2.05 ± 0.36 | 28.9 | 5.11 ± 0.27 | 0.89 ± 0.05 | 3.49 ± 0.09 | 49.2 |
| ALC+SMS50 | 5.21 ± 0.34 | 0.87 ± 0.03 | 3.23 ± 0.07 | 20.7 | 18.1 ± 0.33 | 0.95 ± 0.04 | 14.8 ± 0.30 | 94.8 |
| ALC+SMS75 | 6.23 ± 2.08 | 0.96 ± 0.11 | 5.42 ± 0.53 | 28.7 | 19.6 ± 0.13 | 0.82 ± 0.00 | 9.35 ± 2.86 | 49.5 |
| SJZ | 0.29 ± 0.00 | 0.78 ± 0.02 | 0.21 ± 0.02 | 31.0 | 0.78 ± 0.10 | 1.32 ± 0.05 | 2.54 ± 0.11 | 379 |
| SJZ+SMS2 | 0.49 ± 0.01 | 0.86 ± 0.07 | 0.30 ± 0.01 | 30.8 | 1.18 ± 0.22 | 0.86 ± 0.02 | 1.39 ± 0.10 | 143 |
| SJZ+SMS5 | 0.81 ± 0.20 | 0.79 ± 0.11 | 0.37 ± 0.12 | 21.3 | 2.33 ± 0.03 | 0.81 ± 0.01 | 1.08 ± 0.08 | 62.9 |
| SJZ+SMS10 | 1.48 ± 0.05 | 0.96 ± 0.01 | 1.30 ± 0.11 | 36.5 | 5.82 ± 0.03 | 0.83 ± 0.01 | 3.16 ± 0.68 | 88.8 |
| SJZ+SMS25 | 5.24 ± 0.17 | 0.85 ± 0.01 | 2.99 ± 0.01 | 47.9 | 6.59 ± 0.76 | 1.01 ± 0.04 | 3.88 ± 0.31 | 62.1 |
| SJZ+SMS50 | 6.79 ± 0.07 | 0.83 ± 0.00 | 3.68 ± 0.10 | 22.6 | 7.94 ± 0.04 | 0.86 ± 0.00 | 4.73 ± 0.08 | 29.1 |
| SJZ+SMS75 | 8.26 ± 0.04 | 0.89 ± 0.00 | 5.54 ± 0.12 | 31.5 | 12.1 ± 0.47 | 1.07 ± 0.02 | 15.9 ± 0.13 | 90.3 |
| YEC | 0.58 ± 0.02 | 0.82 ± 0.01 | 0.33 ± 0.05 | 33.1 | | | | |
| YEC+SMS2 | 1.01 ± 0.14 | 0.77 ± 0.01 | 0.43 ± 0.09 | 24.4 | | | | |
| YEC+SMS5 | 1.19 ± 0.02 | 0.78 ± 0.05 | 0.52 ± 0.07 | 23.7 | | | | |
| YEC+SMS10 | 2.81 ± 0.08 | 0.79 ± 0.01 | 1.27 ± 0.01 | 26.0 | | | | |
| YEC+SMS25 | 6.89 ± 0.21 | 0.84 ± 0.00 | 3.84 ± 0.07 | 41.2 | | | | |
| YEC+SMS50 | 6.33 ± 0.17 | 0.88 ± 0.01 | 4.08 ± 0.11 | 33.5 | | | | |
| YEC+SMS75 | 9.77 ± 0.02 | 0.81 ± 0.00 | 4.72 ± 0.27 | 24.5 | | | | |
| SMS | 8.74 ± 0.05 | 0.88 ± 0.01 | 5.64 ± 0.02 | 23.0 | 13.8 ± 0.17 | 1.11 ± 0.02 | 20.0 ± 0.59 | 81.7 |

^a SD, standard deviation (n = 2). ^b Distribution coefficient for a equilibrium concentration of 40 µg mL⁻¹

As seen for non-polar pesticides, the increases in Kd40 for cymoxanil and pirimicarb in soils after amendment compared to unamended soils (Fig. 2) indicated a lower effect of the SMS for the retention of these pesticides in YEC and SJZ soil because of the greater adsorption of pesticides by these unamended soils. The effect of SMS to increase the adsorption of pirimicarb was very low in SJZ soil with a greater clay content, and this is consistent with reports in the literature on the adsorption of pirimicarb by clay minerals (Wei et al. 2001).

The results indicated that a further increase in Kd40 of up to fifteen times for cymoxanil and up to five times for pirimicarb may be achieved when a rate of amendment ≤10% was applied, and higher increases in Kd40 may be achieved in amended soils with doses of SMS >25% for cymoxanil. Increases in the adsorption of pirimicarb were lower than cymoxanil, and in SJZ soil they decreased after amendment at low rates compared to unamended soil, indicating that the SMS effect was not found for polar pesticides in all the soils.

A significant correlation coefficient was found between OC content and adsorption constants Kd40 when the results of both pesticides were considered jointly, although the determination coefficient

r^2 was lower than that found for non-polar pesticides, and accounted for 56.2% of the variance in adsorption according to the equation: $Kd_{40} = 0.201 (0.721) + 0.441 (0.066) OC$. This coefficient improved when DOC and clay content were included in the model ($R^2 = 58.8\%$), and it accounted for up to 71.8% of the variance in adsorption when the OC, DOM, clay and Kow variables were considered according to the equation: $Kd_{40} = -2.713 (1.448) + 0.224 (0.122) OC + 3.707 (2.313) DOC + 0.008 (0.042) \text{ clay} + 2.757 (0.740) Kow$. The adsorption of a polar pesticide was not fully predicted from the increase in OC content by applying different rates of SMS to the soil, as it was for non-polar pesticides. However, the increased adsorption of these hydrophilic pollutants by SMS in amended soils was important despite their lower hydrophobicities, as indicated for other hydrophilic micropollutants adsorbed by organic materials (Nam et al. 2014).

3.3 Estimation and possible prediction of adsorption coefficients of non-polar and polar pesticides for SMS amended soils

A comparison between estimated and experimental adsorption coefficients Kd for the non-polar pesticides tebuconazole and triadimenol and the polar pesticides cymoxanil and pirimicarb for SMS-amended soils were carried out in order to know whether it is possible to predict the Kd adsorption values of pesticides for SMS-amended soils. The Kd values for soils amended with different rates of SMS were estimated from the Kd coefficients obtained by individual components (soil and SMS), as some reports have indicated in the literature (Wang et al. 2010; Chen and Yuan 2011) from the equation: $Kd = f_{sms} Kd_{sms} + f_{soil} Kd_{soil}$, where f_{sms} and f_{soil} are the fractions of SMS and soil for different rates of SMS applied to soils in the different soil-SMS mixtures, and Kd_{sms} and Kd_{soil} are the adsorption distribution coefficients of pesticides for each individual SMS and soil. Linear regressions were then obtained between the Kd estimated and the experimental values for the different pesticides and amended soils assayed.

The results obtained included in Fig. 3 showed a significant correlation between both series of values (estimated and experimental) for non-polar pesticides; r ranged between 0.963 and 0.998 ($p < 0.01$) for tebuconazole and between 0.989 and 0.996 ($p < 0.01$) for triadimenol. Accordingly, these estimated values could be used for predicting the adsorption of these compounds by soils after SMS amendment at different rates from the adsorption data of pesticides by the unamended soil and by SMS. Therefore, the adsorption of non-polar pesticides could be optimized using SMS as a tool to adjust soil adsorption depending on the purpose to be achieved.

However, the correlation coefficients obtained for linear regressions between the estimated and experimental values obtained for polar pesticides (Fig. 3) were lower than those obtained for non-polar pesticides; r ranged between 0.906 and 0.976 ($p < 0.05$) for cymoxanil, and between 0.787 and 0.907 ($p < 0.1$) for pirimicarb. The results indicated a poor match between estimated and experimental adsorption values. The adsorption values estimated from the equations were increased or decreased for cymoxanil and pirimicarb compared to the experimental adsorption values. Different adsorption mechanisms for the retention of non-polar or polar pesticides and amended soils could explain these results.

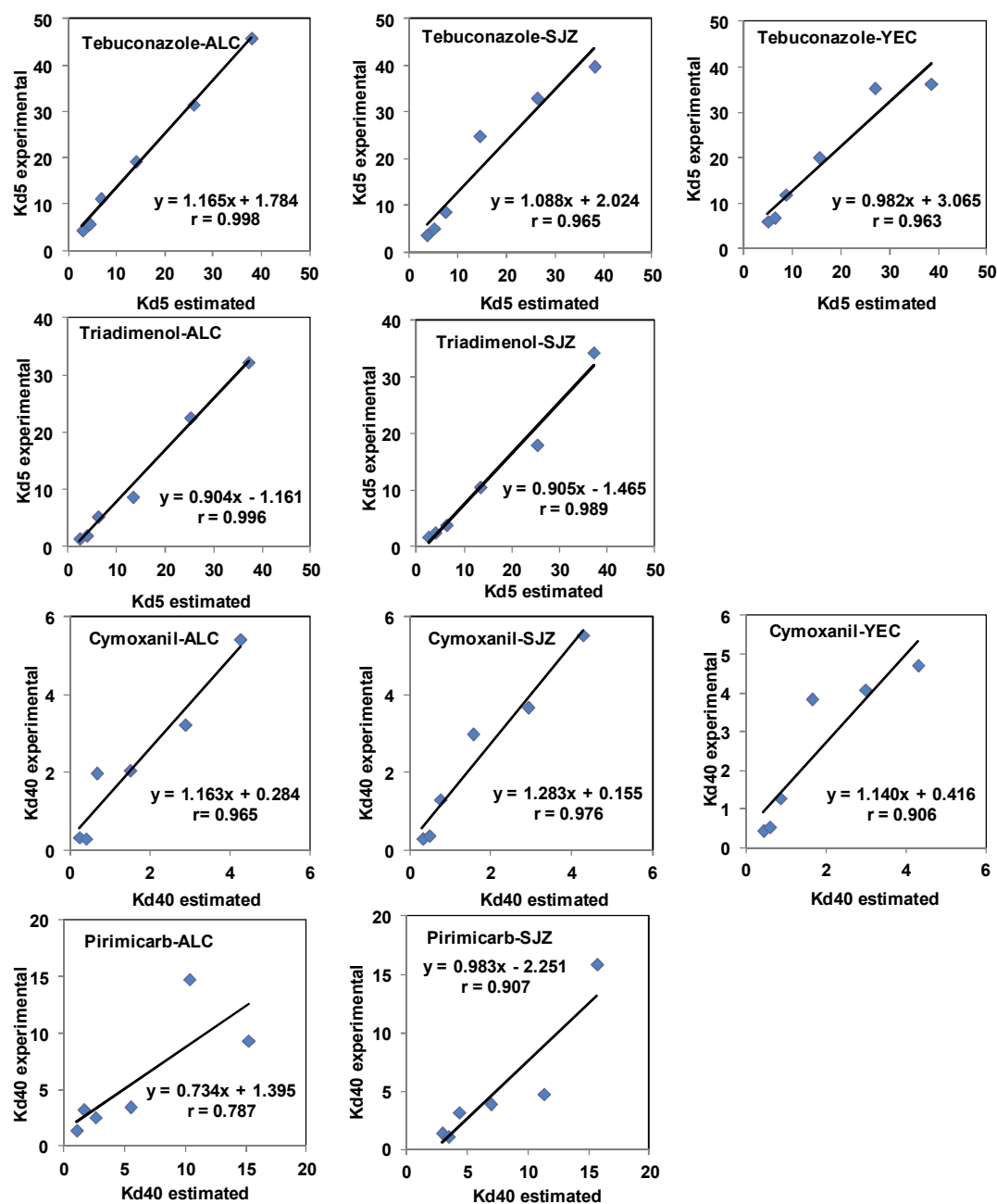


Fig. 3. Linear regressions between estimated and experimental K_d adsorption coefficients of non-polar and polar pesticides by SMS amended soils.

4 CONCLUSIONS

Adsorption of non-polar and polar pesticides by SMS-amended soils increased due to the increase in OC provided by the biosorbent SMS. K_d values increased: i) three-to-five times for tebuconazole and triadimenol and five-to-fifteen times for pirimicarb and cymoxanil for SMS rates $\leq 10\%$ (similar to the agronomic rate) and ii) more than twenty times for non-polar and polar pesticides by applying SMS to soils at rates $\geq 25\%$. The OC content of SMS-soils accounted for more than 90% of the variability in the adsorption of non-polar pesticides, but it accounted for only 56.3% for polar pesticides, indicating the influence of other soil parameters in the adsorption of these polar pesticides. Estimated adsorption results for soils and SMS were consistent with experimental data mainly for non-polar pesticides, and could be used for predicting the adsorption of these compounds by SMS-soils. The results indicate the possibility of using the low-cost biosorbent SMS as a tool for optimizing the adsorption capacities of pesticides by agricultural soils in order to apply the most appropriate biosorbent rates to prevent diffuse or point contamination, and avoid the leaching of pesticides into groundwater.

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Effect of different rates of spent mushroom substrate on the dissipation and bioavailability of cymoxanil and tebuconazole in an agricultural soil

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Abstract

Physicochemical methods to immobilize pesticides in vulnerable soils are currently being developed to prevent water contamination. Some of these methods include the use of different organic residues to modify soils because they could limit the transport of pesticides and/or facilitate their dissipation. Spent mushroom substrate (SMS) may be used for these purposes. Accordingly a study was conducted under laboratory conditions to know the dissipation and bioavailability of the fungicides cymoxanil and tebuconazole over time in a vineyard soil amended with two rates of spent mushroom substrate (SMS) (5% and 50% (w/w)), selected to prevent the diffuse or point pollution of soil. The dissipation of cymoxanil was more rapid than that of tebuconazole in the different soils studied. The dissipation rate was higher in the amended soil than in the unamended one for both compounds, while no significant differences were observed between the amended soils in either case. An apparent dissipation occurred in the amended soil due to the formation of non-extractable residues. Bound residues increased with incubation time for tebuconazole, although a proportion of this fungicide was bioavailable after 303 days. The major proportion of cymoxanil was tightly bound to the amended soil from the start, although an increasing fraction of bound fungicide was bioavailable for mineralization. Soil dehydrogenase activity was significantly affected by SMS application and incubation time; however, it was not significantly modified by fungicide application. The significance of this research suggests that SMS applied at a low or high rate to agricultural soil can be used to prevent both the diffuse or point pollution of soil through the formation of non-extractable residues, although more research is needed to discover the time that fungicides remain adsorbed into the soil decreasing either bioavailability (tebuconazole) or mineralization (cymoxanil) in SMS-amended soils.

Keywords: dissipation, bioavailability, fungicide, soil, amendment rate, pollution

1 INTRODUCTION

Pesticide residues are now being detected in waters and soils in different areas around the world due to the intensive application of these compounds in agriculture (Rabiet et al., 2010; Herrero-Hernández et al., 2013; Pose-Juan et al., 2015a). Consequently, soil and water contamination is a growing concern, as these compounds could be toxic and cause health and environmental problems. European and Spanish legislation (Directive 2009/128/EC and Royal Decree 1311/2012, respectively) on this matter advises introducing specific measures to prevent soil contamination and limit the transport of contaminants through water resources, especially groundwater, to reduce the risks and impacts of pesticide use (EC, 2009; MPR, 2012).

Pesticide application and management in agriculture may cause the diffuse and point pollution of soil and water bodies. Diffuse sources of soil pollution include spray drift, run-off, leaching, etc., whereas

point sources include farmyard activities, direct contamination, and over-spray, among others (Carter, 2000; Balderacchi et al., 2013). Physicochemical methods to immobilize pesticides in vulnerable soils are currently being developed to prevent water contamination. Some of these methods include the use of different organic residues to modify soils, as their high organic matter (OM) content could limit the transport of pesticides from soil to groundwater and/or facilitate their dissipation, avoiding the diffuse or point pollution of waters due to the intensive use of these compounds (Rodríguez-Cruz et al., 2012; Álvarez-Martín et al., 2016).

Spent mushroom substrate (SMS) is an organic residue from mushroom production. In 2012, Spain was the fourth largest producer of mushrooms in Europe, with an output of 146000 t (FAOSTAT, 2015). Mushroom production generates 170000 t of SMS per year (MAGRAMA, 2015). SMS has a high content of OM and nutrients, and can be used as an amendment to improve soil properties and quality (Brunetti et al., 2009; Peregrina et al., 2012). Furthermore, due to its high OM content, SMS could be a useful tool to control the behaviour of pesticides in soils modified with this residue. Previous works have studied the use of SMS to immobilize pesticides in amended soils (Marín-Benito et al. 2012a) or in biobeds (Karanasios et al., 2010; Gao et al., 2015), as well as its effect for controlling the leaching or biodegradation of pesticides (Kadian et al., 2012) and PAHs (García-Delgado et al. 2013).

Cymoxanil and tebuconazole are two fungicides with very different properties that are widely used on vineyards. Previous studies have reported the dissipation of some fungicides in a vineyard soil amended with fresh and re-composted SMS (Marín-Benito et al., 2012b). Furthermore, Herrero-Hernández et al. (2011) have studied the dissipation of tebuconazole in a vineyard soil amended with SMS under field conditions. Both studies highlight the effect of SMS characteristics on the dissipation of fungicides. However, the effect of SMS on the bioavailability and dissipation mechanism of fungicides in amended soil when SMS is applied at two contrasting rates in order to predict their persistence in the soil has not been reported. SMS contrasting rates could be used to prevent diffuse or point soil pollution and results could provide relevant knowledge about these processes. The dissipation of pesticides in soils amended with different rates of other organic residues has hardly been studied (Karanasios et al., 2010), and, in general, a very low range of amendment rates was applied to the soil in these experiments (López-Piñeiro et al., 2013; Sopena and Bending, 2013).

Cymoxanil [1-(2-cyano-2-methoxyiminoacetyl)-3-ethylurea] is an aliphatic nitrogen fungicide that is effective against grape downy mildew. The dissipation of this fungicide has scarcely been studied (Liu et al., 2014). It is considered a non-persistent compound that degrades rapidly, with a time to 50% degradation (DT_{50}) value of 1.2 days in soils under laboratory aerobic conditions (PPDB, 2015). Tebuconazole [(*RS*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl)pentan-3-ol] belongs to the fungicide group of triazoles, which is used to control smut and bunt diseases of cereals and other field crops and powdery mildew in grapevines. Tebuconazole is a hydrophobic fungicide with low solubility in water, and it degrades slowly in soil ($DT_{50} > 365$ days; very persistent), being slightly mobile ($K_{foc}=769$) (PPDB, 2015). Tebuconazole dissipation has been studied mainly in unamended soils (Strickland et al., 2004; Potter et al., 2005; EFSA, 2008b, Fenoll et al., 2011), but there is little information on its dissipation in amended soils (Herrero-Hernández et al., 2011). Tebuconazole and cymoxanil have been detected at concentrations of up to 3.2 µg/L and 0.9 µg/L in surface and ground

waters, respectively, from La Rioja region (Spain), exceeding the EU's $0.1 \mu\text{g L}^{-1}$ limit (Herrero-Hernández et al 2013).

The bioavailability of pesticides in soils and the dissipation mechanism from a mass balance including aqueous and organic extractable fractions and mineralized and non-extractable fractions have been studied in recent years under different environmental conditions and agricultural practices (Mamy et al., 2005, Alonso et al., 2015). However, as far as we know, there are no studies on amended soils under laboratory conditions for cymoxanil and tebuconazole.

The aim of this paper was to study the impact that the SMS applied to soil at two different rates (5% and 50% on a dry weight basis) had on the bioavailability and dissipation mechanism of two fungicides with very different characteristics, namely, tebuconazole and cymoxanil. Studies were therefore carried out on dissipation kinetics (i) and mass balance (ii). Furthermore, soil dehydrogenase activity was assessed (iii) as a soil biochemical parameter to evaluate the impact of organic amendment and fungicides on soil microbial communities, and its possible effect on their dissipation (Muñoz-Leoz et al., 2012; Pose-Juan et al., 2015b). Our findings support the development of strategies for optimizing the dissipation of these fungicides in amended soils in order to restrict the contamination of water by these compounds.

2 MATERIALS AND METHODS

2.1 Pesticides and reagents

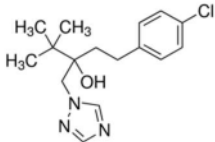
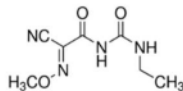
Unlabeled analytical standards of tebuconazole PESTANAL[®] and cymoxanil PESTANAL[®] (>99% purity) were supplied by Sigma-Aldrich Química S.L. (Madrid, Spain). Labeled [triazole- ^{14}C]-tebuconazole and [acetyl 2- ^{14}C]-cymoxanil (specific activities of 4.72 and $10.08 \text{ MBq mg}^{-1}$ and 98.06% and 97.79% purities, respectively) were supplied by IZOTOP Co. Ltd. (Budapest, Hungary). The characteristics of the fungicides are included in Table 1 (PPDB, 2015).

HPLC grade acetonitrile and chloroform anhydrous (>99% purity) were supplied by VWR International Eurolab (Spain). 2,3,5-Triphenyltetrazolium chloride (TTC) and 2,3,5-triphenylformazan (TPF) were supplied by Sigma-Aldrich Química S.L. (Madrid, Spain).

2.2 Organic amendment

Spent mushroom substrate (SMS) from *Agaricus bisporus* cultivation is a pasteurized mixture of wheat straw, poultry manure, urea, and gypsum. This residue was supplied by Sustratos de La Rioja S.L. (Pradejón, Spain). The characteristics of SMS were determined in air-dried samples. The pH was determined in a residue/water suspension (1/2.5 w/v ratio). Organic carbon (OC) content was determined by oxidation (Walkley–Black method). Dissolved organic carbon (DOC) was determined in a suspension of residue in Milli-Q ultrapure water (1/100 w/v ratio), as described by Marín-Benito et al. (2012b). Total N content was determined according to the Kjeldahl method. The organic amendment applied had the following characteristics (on a percentage dry weight basis): pH 6.97, ash content 33.6%, OC content 24.5%, DOC content 1.91%, N content 1.75% and C/N 13.9. Moisture content was 64.5%.

Table 1. Chemical structure and physicochemical properties of fungicides.

| Common name IUPAC name | Chemical structure | WS ^a (mg L ⁻¹) | log Kow ^b | Koc ^c (mL g ⁻¹) | DT ₅₀ ^d (days ⁻¹) | GUS ^e index |
|---|---|--|----------------------|---|--|---------------------------|
| Tebuconazole (<i>RS</i>)-1-p-chlorophenyl-4,4- dimethyl-3-(1 <i>H</i> -1,2,4-triazol- 1-ylmethyl)pentan-3-ol |  | 36 | 3.7 | 769 | 63-365 | 2.0 |
| Cymoxanil 1-[(<i>EZ</i>)-2-cyano-2- methoxyiminoacetyl]-3- ethylurea |  | 780 | 0.0, -0.79 | 43.6 | 0.7-3.5 | -0.37 |

^a Solubility in water at 20 °C, ^b Octanol/water partition coefficient at pH 7-8 and 20 °C, ^c Sorption coefficient normalized to organic carbon content, ^d Time to degradation 50% of compound, ^e Gustafson mobility index of fungicides (Data taken from PPDB 2015).

2.3 Unamended and amended soil characterization

A soil sample (S) was collected from the surface horizon (0-30 cm) of a vineyard located in Sajazarra (La Rioja, Spain). The soil was sieved (<2 mm), and the characteristics were determined by standard analytical methods (MAPA, 1986), and they are included in Table 2. The pH was determined in a soil/water suspension (1/2 w/v ratio), and particle size distribution was determined using the pipette method. The OC and DOC contents in soil extracts (1/2 w/v ratio) in Milli-Q ultrapure water were determined as previously indicated for SMS. Its texture was classified as sandy clay loam.

Table 2. Characteristics of unamended and amended soils.

| | pH | OC (%) | DOC (%) | C/N | Sand (%) | Silt (%) | Clay (%) |
|---------|------|-----------|------------|------|----------|-------------|----------|
| Soil | 7.52 | 0.67 | <0.01 | 11.2 | 67.0 | 11.9 | 21.1 |
| S+SMS5 | 7.26 | 1.73 | 0.06 | | | | |
| S+SMS50 | 7.19 | 16.3 | 0.44 | | | | |

Amended soils were prepared by uniformly mixing soil with SMS at rates of 5% (S+SMS5) and 50% (S+SMS50) w/w, on a dry weight basis. The soils were mixed without sieving (undisturbed), and SMS was also used as received. The initial moisture content of the soils was adjusted to 40% of their maximum water-holding capacity. The unamended and amended soils (10 kg) were incubated in containers outdoors. Soil subsamples were taken one month after the soil had been amended. These samples were air-dried and sieved (<2 mm) prior to their use in the dissipation experiment. Subsamples were analyzed to assess both OC and DOC contents and pH, as previously indicated (Table 2).

2.4 Dissipation studies

The fungicide dissipation experiments (Lynch et al., 1995) were conducted in duplicate. Unlabeled tebuconazole and cymoxanil were added to portions of 500 g of unamended (S) and amended (S+SMS5 and S+SMS50) soils to give a concentration of 2 mg kg⁻¹ dry soil. The soil samples were incubated at 20 °C in the dark. The moisture content of the soil samples was adjusted to 40% of the maximum soil water-holding capacity, and it was maintained by adding sterile Milli-Q ultrapure water when necessary. A sterilized soil sample was also prepared by autoclaving soil at 120 °C for 1 h on three consecutive days. The sterilized unamended soil was treated with each fungicide and incubated as indicated above, and these samples were used as controls to check the chemical degradation of fungicides. Finally, soils for microbiological control were prepared by adding only sterile Milli-Q ultrapure water. All the soils were thoroughly stirred with a sterilized spatula, and all the steps were performed in a sterile cabinet. Sampling was performed at different times, and fungicide was extracted from the soil at different time intervals (up to 303 days), depending on each compound's degradation rate.

Simultaneous incubations were carried out with ¹⁴C-labeled fungicides to study the dissipation mechanism and bioavailability of pesticides. Aqueous solutions of an appropriate concentration of an unlabeled fungicide were labeled with ¹⁴C-fungicides, and a volume of 10 mL of these solutions was added to 500 g fresh weight of unamended (S) or amended (S+SMS5 and S+SMS50) soils to give a concentration of 2 mg kg⁻¹ dry soil, and an activity of approximately 100 Bq g⁻¹. In these soil samples, a ¹⁴CO₂ trap, consisting of a scintillation vial containing 1 M NaOH (1 mL), was attached to the lid via a stainless steel clip (Reid et al., 2002).

2.5 Extraction and determination of cymoxanil and tebuconazole

At each sampling time, 2 × 5 g of each duplicate treatment were shaken in glass test tubes at 20 °C for 24 h, with methanol (10 mL) for tebuconazole and methanol/water 50:50 (10 mL) for cymoxanil. The samples were then centrifuged at 5045 g for 15 min, and the fungicide extracts were filtered in a Minisart NY 25 filter (Sartorius Stedim Biotech, Germany) to remove particles > 0.45 µm. For the determination of the fungicides, a volume of the extract (5 mL) was transferred to a clean glass test tube and evaporated until dry at 25 °C under a nitrogen stream using an EVA-EC2-L evaporator (VLM GmbH, Bielefeld, Germany). The residue was dissolved in 0.5 mL of acetonitrile and then transferred to a glass vial for analysis. The recoveries of the extraction method were determined by spiking three unamended and amended soil samples with analytical grade fungicide to a final concentration of 2 mg kg⁻¹, performing the extraction procedure as described above. The mean recovery values varied between 70% and 111% for both the fungicides studied.

Tebuconazole and cymoxanil were determined by HPLC-MS using a Waters chromatograph (Waters Assoc., Milford, MA, USA) with a Phenomenex Luna (3µ C18, 150 x 4.60 mm) column. The mobile phase was 70:30 acetonitrile:water for cymoxanil, and 90:10 acetonitrile:water+0.1% formic acid for tebuconazole. The flow rate was 0.3 mL min⁻¹ for cymoxanil and 0.4 mL min⁻¹ for tebuconazole, and the sample injection volume was 10 µL for cymoxanil and 20 µL for tebuconazole. The MS parameters were as follows: capillary voltage, 3.1 kV; source temperature, 120°C; desolvation temperatures and

desolvation gas flow set at 300°C and 400 L h⁻¹, respectively, and cone gas flow set at 60 L h⁻¹. The positive molecular ion (*m/z*) was 199.19 for cymoxanil and 308.80 for tebuconazole. Retention times for cymoxanil and tebuconazole were 7.9 and 6.2 min, respectively. Monitoring also involved positive molecular ions (*m/z*) 128.09 and 216.2 for cymoxanil metabolites (cyano(methoxyimino)acetic acid and 3-ethyl-4-(methoxyamino)-2,5-dioximidazolidine-4-carboxamide, respectively), and 168, 240, 224 and 322 for tebuconazole metabolites (3,3-dimethyl-1-([1,2,4]triazol-1-yl)-butan-2-one, 4-hydroxy-5,5-dimethyl-4-(1*H*-1,2,4-triazol-1-ylmethyl)hexanoic acid, 5-*tert*-buthyl-5-(1*H*-1,2,4-triazol-1-ylmethyl)hexanoic acid and 1-(4-chlorophenyl)-3-hydroxy-4,4-dimethyl-3-([1,2,4]triazol-1-ylmethyl)-pentan-1-one). The formation of metabolites during the dissipation experiment was only qualitatively monitored because metabolite standards were not available. The method validation was described previously (Pose-Juan et al., 2014). The limit of detection (LOD) and quantification (LOQ) values obtained were 0.022 and 0.069 µg L⁻¹ for cymoxanil, and 0.012 and 0.035 µg L⁻¹ for tebuconazole.

2.6 Extraction and determination of ¹⁴C-cymoxanil and ¹⁴C-tebuconazole

The extraction of the ¹⁴C-pesticides from the soil was carried out in two sequential steps: initially, 2 × 5 g of each duplicate treatment were extracted with 10 mL of a 0.01 M CaCl₂ Milli-Q ultrapure water solution for 24 h, and then a second extraction with 10 mL of the organic solvent methanol was carried out for 24 h.

The quantitative determination of ¹⁴C-tebuconazole and ¹⁴C-cymoxanil after extraction was performed by liquid scintillation using a Beckman LS 6500 liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA). The radioactivity of the solution was measured in disintegrations per minute (dpm), being determined in duplicate in 1 mL of aqueous methanol extract to which 4 mL of scintillation cocktail was added (Ecoscint TMA, National Diagnostics, Atlanta, GA).

The residues of ¹⁴C-fungicides remaining in the soil after extraction were determined by the combustion of triplicate 1 g dried soil samples, using a Biological Oxidizer (RJ. Harvey OX-500 Instrument Corporation, NJ) under O₂ excess at 900 °C. The ¹⁴CO₂ generated was trapped in a mixture of ethanolamine (1 mL) and scintillation cocktail (Oxysolve C-400, Zinsser Analytic, Berkshire, UK, 15 mL), and determined as indicated above. ¹⁴CO₂ from mineralized ¹⁴C-fungicides in the scintillation vial containing 1 M NaOH (1 mL) was determined at the different sampling times by mixing with 4 mL of scintillation cocktail, and determined as previously indicated.

2.7 Soil dehydrogenase activity

Soil dehydrogenase activity (DHA) was determined following the Tabatabai method (Tabatabai, 1994) at different times after fungicide application.

2.8 Data analysis

The degradation kinetics for the fungicide was fitted to a single first-order (SFO) kinetic model ($C = C_0 e^{-kt}$) or first order multi-compartment (FOMC) model ($C = C_0 / ((t / \beta) + 1)^\alpha$), known also as the Gustafson and Holden model. *C* is the fungicide concentration at time *t*, *C*₀ is the initial fungicide concentration, *k* (day⁻¹) is the degradation rate, *α* is a shape parameter determined by the coefficient of

variation of k values, and β is a location parameter. For the selection of the kinetic model that best describes the degradation results, FOCUS work group guidelines were followed (FOCUS, 2006). The coefficient of determination (r^2) and the chi-square (χ^2) test were calculated as indicators of the goodness of fit. The time to 50% degradation, or DT_{50} value, was used to characterize the decay curves and compare variations in degradation rates. The parameters of the kinetic models were estimated using the Excel Solver add-in Package (FOCUS, 2006).

Analysis of variance (ANOVA) was used to evaluate the effects of the different treatments on the dissipation of fungicides. Standard deviation (SD) was used to indicate variability among replicates, and the least significant difference (LSD), at a confidence level of 95%, was determined to evaluate the effects of different soil treatments on DT_{50} values and dehydrogenase activity. Statgraphics Plus version 5.1 statistical software (Statgraphics Plus Corp., Princeton, NJ) was used.

3 RESULTS AND DISCUSSION

3.1 Dissipation of tebuconazole and cymoxanil in unamended and amended soils

The dissipation kinetics of tebuconazole and cymoxanil (expressed as a percentage of the amount of fungicide initially applied versus the incubation time) in an unamended soil and a soil amended with SMS at rates of 5% and 50% are included in Fig. 1. At the end of the incubation time (303 days for tebuconazole and 15 days for cymoxanil), the dissipation of tebuconazole ranged between 34.8% and 76.7%, and cymoxanil was fully dissipated in the different soil samples.

The dissipation pattern of both fungicides was fitted to SFO or FOMC models, and kinetic parameters were calculated for each fungicide and soil treatment (Table 3). The dissipation kinetics of tebuconazole in both the unamended and S+SMS5 soils fitted the FOMC model better, whereas in the S+SMS50 it fitted the SFO model better. In turn, the dissipation kinetics of cymoxanil in all the soils studied fitted the SFO model better. Previous works have reported the dissipation curves of tebuconazole in unamended and amended soils fitted to a SFO or a biphasic model (Strickland et al., 2004; Potter et al., 2005; Herrero-Hernández et al., 2011). The dissipation kinetics of cymoxanil in unamended soils was fitted to SFO or FOMC models in previous studies (EFSA, 2008a).

Table 3. Kinetics parameters for the dissipation of tebuconazole and cymoxanil in unamended and SMS amended soils.

| | SFO model | | FOMC model | | | | | | |
|---------------------|--------------------------|------------------|------------|----------|--------------------|--------------------|------------------|-------|----------|
| | k (day ⁻¹) | DT_{50} (days) | r^2 | χ^2 | α | β | DT_{50} (days) | r^2 | χ^2 |
| Tebuconazole | | | | | | | | | |
| Soil | 0.001 | 592±119 | 0.78 | 6.0 | 0.06 | 0.60 | > 1000 | 0.91 | 3.6 |
| S+SMS5 | 0.005 | 141±9.26 | 0.93 | 5.9 | 1.25 | 1.70×10^2 | 126±29.8 | 0.93 | 5.5 |
| S+SMS50 | 0.004 | 168±18.2 | 0.90 | 10.6 | 1.03×10^3 | 2.54×10^5 | 168±21.1 | 0.90 | 10.9 |
| Cymoxanil | | | | | | | | | |
| Soil | 0.90 | 0.8±0.0 | 0.99 | 6.4 | 6.74×10^4 | 7.50×10^4 | 0.8±0.0 | 0.99 | 6.8 |
| S+SMS5 | 1.90 | 0.4±0.0 | 0.99 | 5.4 | 1.24 | 0.30 | 0.2±0.0 | 0.98 | 5.9 |
| S+SMS50 | 1.62 | 0.4±0.1 | 0.99 | 1.0 | 6.64×10^2 | 4.10×10^2 | 0.4±0.1 | 0.98 | 1.7 |

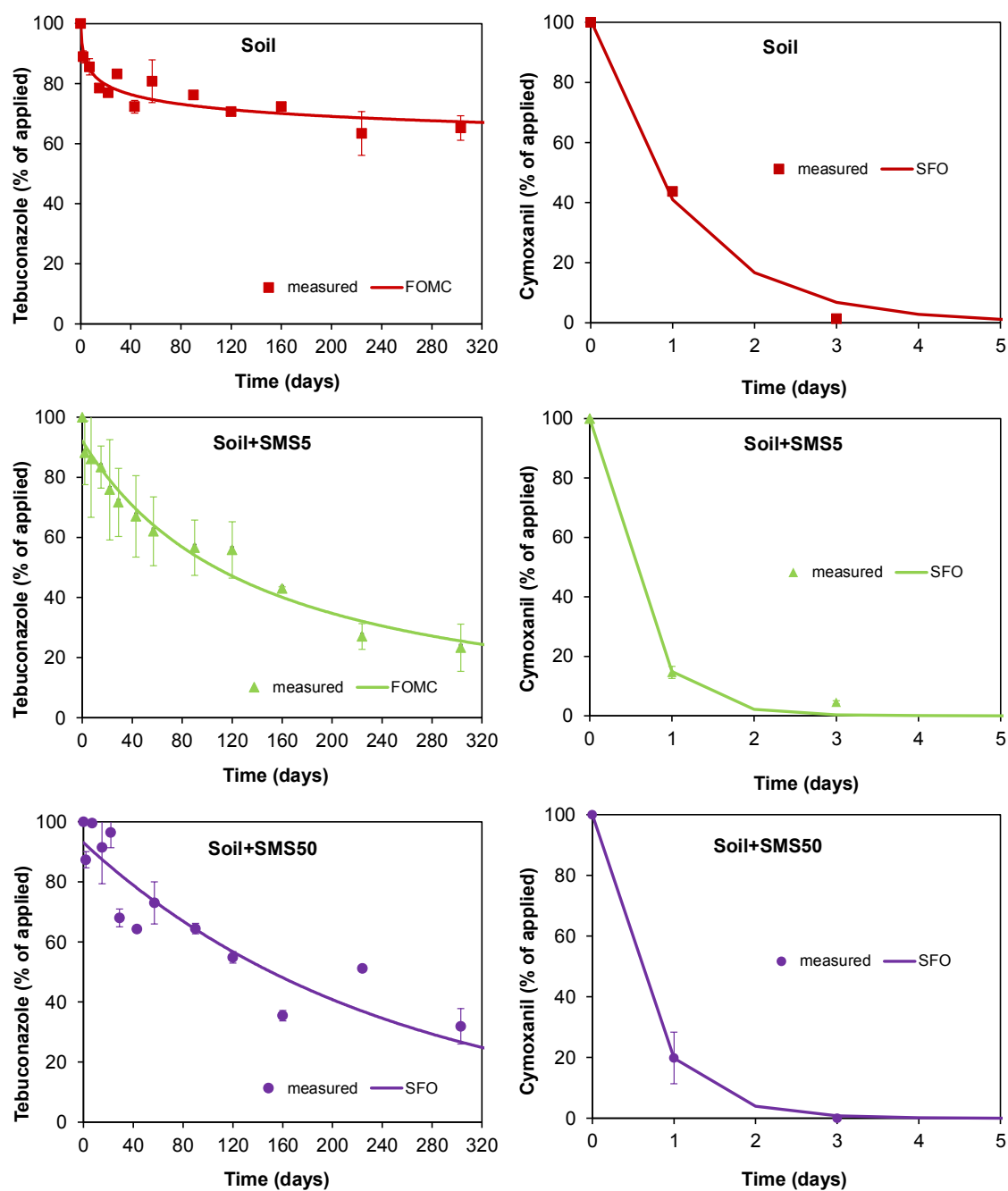


Fig. 1. Degradation kinetics of tebuconazole and cymoxanil in unamended soil and soil amended with SMS at rates of 5% and 50%. Bars indicate the standard deviation of the mean value (n=4)

Cymoxanil dissipation was very rapid (< 1 day) in both the unamended and amended soils. The DT_{50} values between 0.4 and 0.8 days were consistent with the range of values reported for this fungicide in soils under similar conditions as in this study, which varied between 0.2-7.3 days (PPDB, 2015), 0.1-4.3 days (EFSA, 2008a) or < 1 day (Liu et al., 2014). The dissipation rate of cymoxanil increased when SMS was applied to the soil, although cymoxanil adsorption of fungicide by the amended soils increased. The adsorption constant of cymoxanil by the unamended soil ($K_f=0.46$) was lower than those by amended soils ($K_f=0.81$ and 6.79 , respectively by S+SMS5 and S+SMS50) (Alvarez-Martin et al., 2016). The influence of adsorption on decreasing the dissipation rate of pesticides in soils due to a decrease in the bioavailability and biodegradation of organic compounds adsorbed by the soil has not been observed for cymoxanil, although it has been reported for other pesticides, such as diazinon and myclobutanil (Marín-Benito et al., 2014), or fungicides, such as penconazole, metalaxyl and iprovalicarb (Marín-Benito et al., 2012a) in SMS-amended soil, but changes in cymoxanil dissipation rates were not very relevant between the soils amended at 5% and 50% of SMS.

The DT_{50} values for the dissipation of tebuconazole were higher than those for cymoxanil in all the soils studied (Table 3). DT_{50} in the unamended soil (> 1000 days) was higher than that reported in previous works (from 43 to 693 days) for agricultural soils under laboratory conditions (Strickland et al., 2004; Potter et al., 2005; EFSA, 2008b; Fenoll et al., 2011; PPDB 2015). However, the tebuconazole dissipation rate increased significantly in the SMS-amended soils. DT_{50} values were 126 and 168 days for 5% and 50% SMS-amended soils, respectively). They decreased up to 293 times compared to the unamended soil, but the SMS rate had no significant effect on fungicide dissipation. The lower adsorption coefficients of tebuconazole by the unamended soil ($K_f=3.06$) than by the SMS-amended soils ($K_f= 6.24$ and 36.6 by S+SMS5 and S+SMS50, respectively) (Álvarez-Martín et al., 2016) could not explain the higher residual amount of fungicide found at the end of incubation, and the higher persistence of fungicide in the unamended soil than in the amended one. These results could be supported by considering that (i) the application of organic residues such as SMS to soil can increase the soil microbial biomass, its microbial activity, and its capacity to biodegrade the pesticide (Karanasios et al., 2010), leading to higher dissipation, or (ii) the increase in soil OM due to the application of the amendment could help to enhance pesticide adsorption and the formation of non-extractable residues over time (Kim et al., 2003; Marín-Benito et al., 2012b), which could explain the more rapid apparent dissipation of tebuconazole in amended soils. Similar results have been reported for other hydrophobic pesticides such as linuron in soils amended with different organic residues (sewage sludge, grape marc, or SMS) (Marín-Benito et al., 2014). Some authors have reported that the persistence of pesticides depends on the dissipation mechanism of these compounds in amended soils (Kim et al., 2003).

The degradation of fungicides in sterilized soil was slower than in non-sterilized soil (data not shown), which indicates that the soil microbial community played an important role in fungicide degradation. In sterilized soil, the percentage of fungicide degraded at the end of the incubation period was 18.7% for tebuconazole and 74.6% for cymoxanil. Previous papers have reported that the degradation of tebuconazole is mediated mainly by microorganisms (Bending et al., 2007; Muñoz-Leoz et al., 2011; Li et al., 2015). No information about the biodegradation of cymoxanil is available in the literature.

During the incubation experiments, some metabolites of tebuconazole and cymoxanil were qualitatively monitored. The monitoring of the formation of tebuconazole metabolites (peak area) over time in the soil extracts from unamended and amended soils is presented in Fig. 2. Two degradation products from tebuconazole, 3,3-dimethyl-1-([1,2,4]triazol-1-yl)-butan-2-one and 1-(4-chlorophenyl)-3-hydroxy-4,4-dimethyl-3-([1,2,4]triazol-1-ylmethyl)-pentan-1-one (molecular weights 167 and 321, respectively) were detected in the soil extracts up to 303 days. These metabolites were previously detected in other degradation studies on tebuconazole under laboratory or field conditions (Herrero-Hernández et al., 2011; Potter et al., 2005; Strickland et al., 2004). Both metabolites steadily increased with time between 90 and 160 days for the unamended or SMS-amended soils, and then their formation decreased at the end of the experiment. Metabolite formation was higher in the soil treated with a low rate of SMS than in the one treated with a high rate. The highest rate of amendment increased tebuconazole retention, initially protecting the fungicide from degradation.

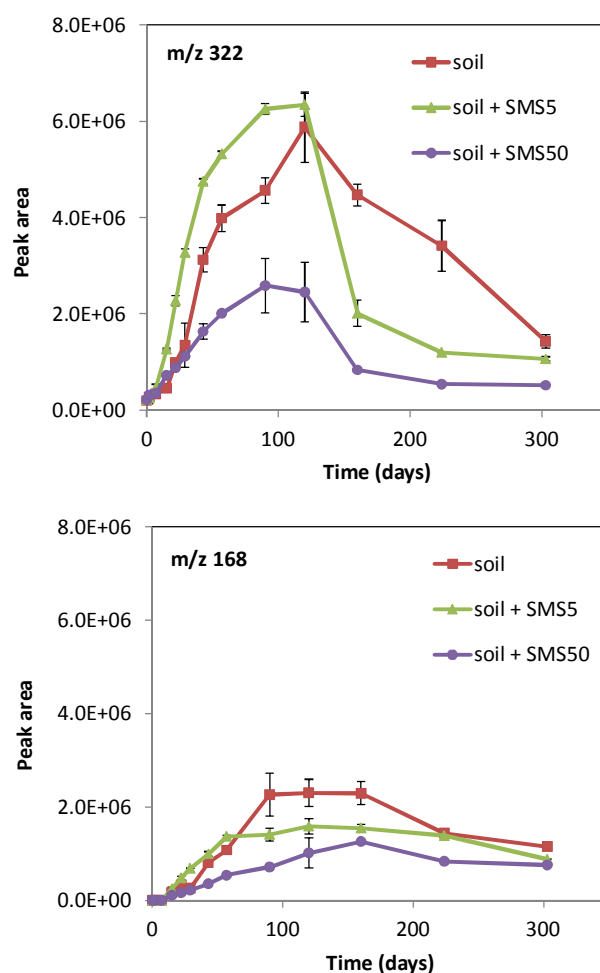


Fig. 2. Peak areas of tebuconazole metabolites ($m/z=322$ and 168) over time in unamended soil and soil amended with SMS at rates of 5% and 50%. Error bars indicate standard deviation of the mean value ($n=4$).

Traces of the cymoxanil metabolite, cyano(methoxyimino)acetic acid (molecular weights 127) were also detected in the soil extracts up to seven days, although no differences in its detection between soils were observed. It has been reported that cymoxanil metabolites degrade very quickly in soil (EFSA, 2008a).

3.2 Mass balance of ^{14}C -tebuconazole and ^{14}C -cymoxanil in unamended and amended soils and dissipation mechanism

The total ^{14}C balance for ^{14}C -tebuconazole and ^{14}C -cymoxanil corresponding to mineralized, CaCl_2 -extracted, methanol-extracted, and non-extractable (bound residues) fractions of ^{14}C -tebuconazole and ^{14}C -cymoxanil in unamended and SMS-amended soils over time is shown in Fig. 3. The total mass balance (expressed as a percentage of the ^{14}C initially applied) was as follows: $94 \pm 3\%$, $100 \pm 5\%$, $112 \pm 5\%$ for ^{14}C -tebuconazole, and $90 \pm 1\%$, $86 \pm 1\%$, and $92 \pm 2\%$ for ^{14}C -cymoxanil in the unamended and S+SMS5 and S+SMS50 soils, respectively.

The mineralization of cymoxanil was fast after six days, and there was a steady increase in $^{14}\text{CO}_2$ evolution during the incubation time studied (up to 102 days). Cymoxanil was ^{14}C -labeled in the 2-acetyl group, with the rapid $^{14}\text{CO}_2$ evolution indicating that this group degraded very quickly. The mineralization of ^{14}C -cymoxanil was always much higher than that of ^{14}C -tebuconazole. Tebuconazole was ^{14}C -labeled in the triazole ring, and mineralization increased slowly up to 303 days, indicating that this triazole group is less accessible for microorganisms to be mineralized. These results are consistent with the dissipation rates of both fungicides.

The mineralization of ^{14}C -cymoxanil and ^{14}C -tebuconazole was significantly higher in the unamended soil compared to the SMS-amended soils. The amounts of ^{14}C -cymoxanil mineralized after 102 days were 59.3%, 57.8% and 38.5% in the unamended, S+SMS5 and S+SMS50 soils, respectively. For ^{14}C -tebuconazole, the amounts mineralized were 3.37%, 1.13% and 0.19% in the unamended, S+SMS5 and S+SMS50 soils, respectively, after 303 days of incubation. The higher adsorption of the fungicides by the amended soils, as previously indicated, could explain these results, especially in the soil amended with the highest SMS rate. The influence of adsorption on decreasing mineralization has been reported for other pesticides in unamended and amended soils (Houot et al., 1998; Kim et al., 2003; Marín-Benito et al. 2012b, 2014).

The high mineralization of cymoxanil (60.4% in unamended soil) has been reported (EFSA, 2008a). The low mineralization of tebuconazole was similar to that for other triazole fungicides, such as propiconazole (Kim et al., 2003; Barriuso et al., 2008) or penconazole (Marín-Benito et al., 2012b), being explained by the difficulty microorganisms have to degrade the triazole ring, where ^{14}C is labeled. In SMS-amended soils, the amount of $^{14}\text{CO}_2$ evolved was below the impurity level, so the true mineralization of ^{14}C -tebuconazole may be lower than indicated. A very low mineralization ($<0.1\%$) of ^{14}C -tebuconazole has been observed in a previous report (EFSA, 2008b).

The extracted amounts of both ^{14}C -fungicides in 0.01M CaCl_2 and methanol decreased with incubation time in the unamended and SMS-amended soils. According to Alonso et al. (2015), fungicide degradation and the formation of bound residues could explain the decrease in extractability with incubation time.

For tebuconazole, the ^{14}C amounts extracted with a water solution were initially higher in the unamended soil (40.2%) than in the amended ones (11.5%-23.6%), and they were lower in S+SMS50 than in S+SMS5, indicating the effect of the SMS rate in the water extractable fraction. These amounts decreased over time, but after 303 days they were still higher in the unamended soil. These results are related with the adsorption coefficient of tebuconazole, which is higher for the SMS-amended soils, indicating a higher bioavailability of the fungicide in the unamended soil than in the amended ones. However, the amounts of ^{14}C -tebuconazole extractable with methanol followed the order: S+SMS50 (67.1%) > S+SMS5 (62.0%) > S (45.5%), but the decrease over time was higher in the SMS-amended soils compared to the unamended soil according to the highest dissipation rate of fungicide in the amended soils (Fig. 1, Table 3). At the end of the incubation period, the extracted ^{14}C amounts were higher in the unamended soil (36.3%) compared to the SMS-amended soils (25.2%-28.6%).

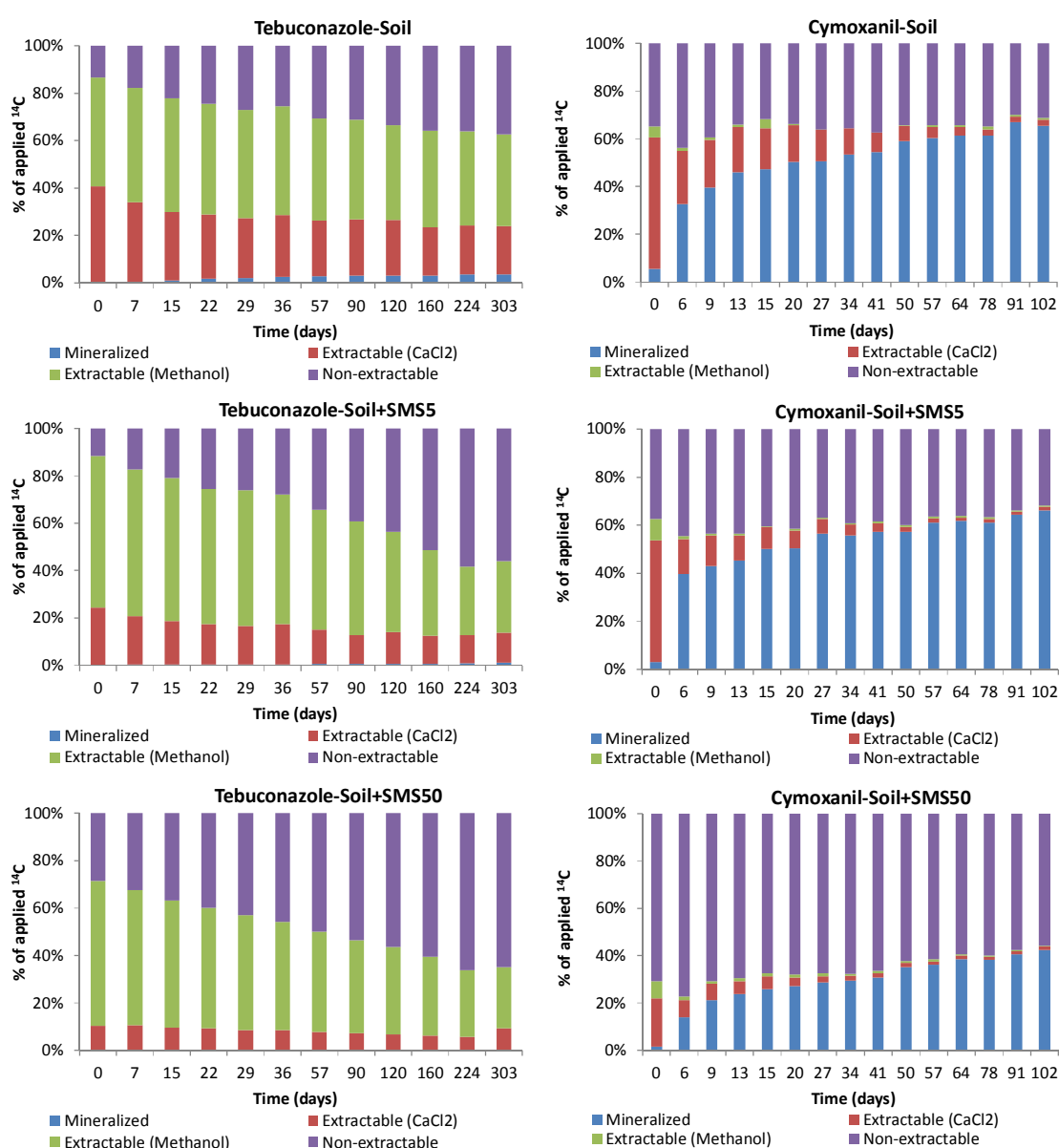


Fig. 3. Mineralized, CaCl₂-extractable, methanol-extractable and non-extractable fractions of ^{14}C -tebuconazole and ^{14}C -cymoxanil in unamended and SMS-amended soils.

The water extractable ^{14}C residues for cymoxanil were initially higher than for tebuconazole, in agreement with its potential bioavailability, and they decreased over time. These amounts were higher in the unamended soil (48.2%), and decreased when the SMS rate applied to the soil increased (43.7%-19.1%). At the end of the incubation period, the water extractable ^{14}C residues decreased in all the soils, ranging between 1.19% and 2.25%. The methanol extracts for ^{14}C -cymoxanil were initially higher (6.70%-7.77%) in the SMS-amended soils than in the unamended one (3.96%), and decreased quickly in all the soils, with the extracted amounts being $< 1\%$ after nine days. The water and methanol extractable ^{14}C amounts correspond to cymoxanil and its metabolites formed during degradation, and these results were consistent with the higher extraction of labeled fungicide compared to non-labeled cymoxanil.

The amounts of non-extractable residues in the soils were initially lower for tebuconazole than for cymoxanil. These amounts increased with incubation time for tebuconazole, whereas a decrease was observed for cymoxanil. The formation of non-extractable ^{14}C -cymoxanil residues decreased with incubation time due to an increase in the $^{14}\text{CO}_2$ evolved. Non-extractable residues could be available for degradation or mineralization due to re-equilibration between the sorbed and soluble phases (Alonso et al., 2015). However, the formation of bound residues for tebuconazole continued throughout the incubation period, indicating a continuous incorporation of new residues in the non-extractable residues pool, and greater physicochemical interactions between the fungicide and the soil, as reported for other pesticides in previous works (Barriuso et al., 2008; Fenlon et al., 2011; Marín-Benito et al., 2014; Alonso et al., 2015).

The percentages of bound residues of cymoxanil after 102 days were similar in both the unamended and S+SMS5 soils (28.2% and 27.8%, respectively) and increased in S+SMS50 (50.5%). For tebuconazole, these percentages increased in the SMS amended soils (53.2% and 63.7% in S+SMS5 and S+SMS50, respectively) compared to the unamended soil (35.2%) after 303 days of incubation. This is consistent with the higher dissipation rate of tebuconazole in the amended soils compared to the unamended one (Table 3), with the formation of bound residues decreasing its bioavailability. The non-extractable residues could come from the parent compounds or metabolites of fungicides, as shown for other pesticides (Marín-Benito et al., 2014), and it seems to be controlled by the soil OC content (Mamy et al., 2005).

3.3 Effect of SMS and fungicides on soil dehydrogenase activity

Dehydrogenase activity (DHA) was analyzed for the unamended and amended soils, either untreated or treated with fungicides during the dissipation period, as a useful indicator of overall microbial activity in soil to evaluate the impact of disturbances on soil quality (García-Izquierdo et al., 2003).

The DHA values obtained were dependent upon the amendment amount and incubation time for both fungicides studied (Fig. 4). The addition of SMS at different rates to the soil significantly increased the DHA values ($p < 0.001$), showing the stimulatory effect of the amendment on soil microbial activity, as previously reported (Kadian et al., 2012; Marín-Benito et al., 2014). Soil DHA follows the order $S < S+\text{SMS5} < S+\text{SMS50}$ for the untreated soils or those treated with both fungicides. Gao et al. (2015) have reported higher activities in SMS-biomixtures due to a more readily available carbon. However, DHA

decreased significantly with incubation time in all the soils and treatments studied ($p < 0.001$ and $p < 0.05$ for soils treated with tebuconazole or cymoxanil, respectively). This decrease in DHA recorded in the soils treated with both fungicides over time is because the fungicides were dissipated with incubation time, and they were less available for microorganisms.

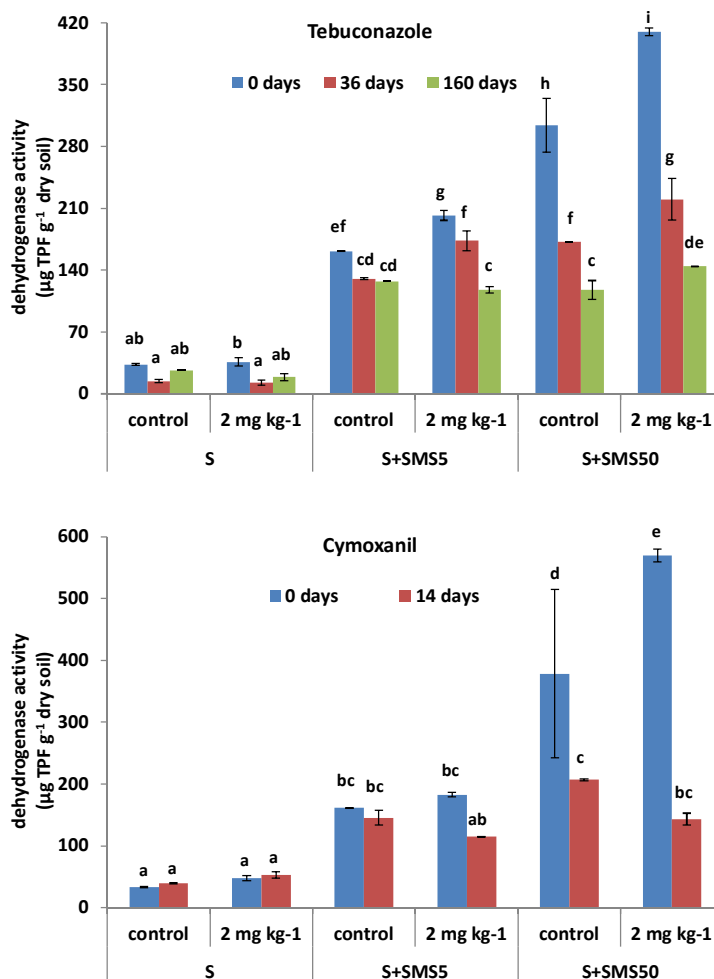


Fig. 4. Soil dehydrogenase activity for unamended (S) and SMS-amended soils (S+SMS5 and S+SMS50), untreated (control) and treated with tebuconazole or cymoxanil (2 mg kg⁻¹) at different sampling times. Bars indicate the standard deviation of the mean (n=4). Different letters indicate significant differences ($p < 0.05$), considering effects of amendment, fungicide and incubation time.

The effect of fungicide on soil DHA was not significant ($p > 0.1$) for either fungicide treatment when considering all the soils together. At the beginning of incubation, the DHA values in the SMS amended-soils treated with tebuconazole were higher than the control soils (without fungicide), indicating that soil microbial activity was stimulated by an extra carbon source provided by the addition of pesticides and amendment to the soil (Herrero-Hernández et al., 2011; Muñoz-Leoz et al., 2013). This

effect was also observed for cymoxanil, but only in the soil amended with the highest rate of SMS. Previous works, nevertheless, have reported that tebuconazole leads to a reduction in microbial activity, probably due to its negative effect on soil fungal communities (Muñoz-Leoz et al., 2011; Cycon et al., 2006).

4 CONCLUSIONS

The results obtained in this work reveal changes in the dissipation and bioavailability of tebuconazole and cymoxanil in soil amended with SMS. The dissipation rate of both fungicides was lower in the unamended soil than in both the SMS-amended soils. The apparent increase in the dissipation rate of both compounds in amended soils was due to an increase in the adsorption by these soils in an unavailable form. Bound residues of tebuconazole increased in all the soils over incubation time, although its bioavailability was lower in the SMS-amended soils. Bound residues of cymoxanil were higher in the SMS-soil, and they decreased over incubation time in all the soils because cymoxanil was mineralized from the start of the incubation period. The mineralization of ^{14}C -tebuconazole and ^{14}C -cymoxanil was higher in the unamended soil compared to the SMS-amended soils due to the higher fungicide retention by the amended soils. Therefore, SMS applied at different rates to soil revealed a potential for designing strategies to prevent the diffuse or point contamination of soils because it could reduce the extractable fraction of fungicides through the formation of non-extractable residues. However, soil particles containing these less-extractable residues of fungicides may be mobilized and transported into aquatic ecosystem where they can be released and other processes such as fungicide leaching or runoff should be taken into account to have a further view of the effect of the proposed soil amendment strategy.

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Leaching of two fungicides in spent mushroom substrate amended soil: Evaluation of different factors influencing their mobility

Manuscript

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Abstract

A study to know the leaching of two fungicides, tebuconazole and cymoxanil, in a soil amended with spent mushroom substrate (SMS) and evaluate how different factors influence this process was conducted. The objective was supported by the potential use of SMS as a biosorbent to immobilize pesticides in vulnerable soils and the need to know how it could affect the subsequent transport of these retained compounds. Breakthrough curves (BTC) were obtained for ^{14}C -fungicides non incubated and incubated over 30 days on the columns packed with an unamended soil (S) and this soil amended with SMS at rates of 5% (S+SMS5) and 50% (S+SMS50) under saturated and saturated-non saturated flows. The higher leaching of tebuconazole (>50% of the total ^{14}C added) was found in S when saturated water flow was applied to the column, but this amount decreased when a saturated-non saturated flow was applied and when the soil was amended with SMS at two rates. However, a significant decrease in the leaching was denoted for tebuconazole after incubation in the column, especially in S+SMS50 when two flows were applied. Besides cymoxanil leaching was total in S and S+SMS when a saturated flow was applied with maximum peak concentrations close to 1 PV, although BTCs showed peaks of lower concentrations in S+SMS. Retained amounts only increased in S+SMS when a saturated-non saturated flow was applied and a more relevant effect of SMS to decrease leaching of cymoxanil was observed when it was previously incubated on the column, although mineralization was enhanced in this case. The results highlighted the effect of SMS to control leaching of fungicides with different characteristics under different flow conditions. The retention enhancement of these compounds by SMS after fungicide aging in the soil helps to decrease the leaching of tebuconazole or decrease the mineralization of cymoxanil.

Keywords: Leaching, soil column, tebuconazole, cymoxanil, spent mushroom substrate, saturated flow, saturated- non saturated flow, mineralization

1 INTRODUCTION

The main European crop producers have increased the use of fungicides in the last years. Thus in 2010, 42726 tonnes of fungicide were applied in Italy, 29829 in France, 10153 in Spain, 10087 in Germany and 5320 in the UK (FAOSTAT, 2015). In Spain it means a cost of 171.43 millions of euro (MAGRAMA, 2015). Although synthetic fungicides are highly effective, their repeated use may led to problems such as environmental pollution, development of resistance, and/or residual toxicity (Komárek et al., 2010). In vine cultivation, the application of fungicides is usually intensive and the presence of those compounds and their residues has been detected in soils and waters from some vineyard areas in last years (Papadakis et al., 2015; Peyrard et al., 2015; Pose-Juan et al., 2015).

Due to increasingly stringent restrictions about the limit of pesticides in waters (Directive No. 2008/105/EC, amending the Water Framework Directive 2000/60/EC), it is necessary to develop new tools to avoid pesticides could reach the groundwater. Adsorption is one of the most efficient techniques for preventing water pollution or for polluted water treatment over the last decades. The interest in the use of low-cost adsorbent materials has lately led researchers to explore the adsorption capacity of organic wastes (Gupta et al., 2009; Kurniawan et al., 2006). Although they are expected to have less adsorption capacity than the synthetic adsorbents, their low cost makes them competitive alternatives (Kyzas and Kostoglou, 2014). Some of these cheaper biomaterials are originated from agricultural activities (manure, biosolids, biochar) or from industrial activities (mushroom farming, wine and olive oil production) and they are characterized by their high organic carbon (OC) content. By this fact their simultaneous use as organic amendments of soil to increase agricultural productivity (Courtney and Mullen, 2008; Udom et al., 2016) and as adsorbents to control the adsorption of organic contaminants in soils is not surprising taking into account OC is one of the most important soil factors influencing this process (Tran et al., 2015; Zolgharnein et al., 2011).

Studies on the use of organic residues with potential to increase the adsorption of pesticides by soils have been extensively reported in the literature (Marín-Benito et al., 2012; Rodríguez-Cruz et al., 2012). However, the influence of these organic residues on the mobility of adsorbed pesticides in the soil profile has been less explored. In general, investigations have been carried out on herbicides (phenylureas, triazines) (Fernández-Bayo et al., 2015; Navarro et al., 2012; Peña et al., 2016), and some of them have also included different classes of pesticides (Larsbo et al., 2013), and they were carried out under similar flow conditions and state of pesticide despite these factors together the characteristics of pesticides may determine to a greater or lesser extent the mobility of these compounds and the possible contamination of groundwaters. Recent studies have also handled mathematical models to elucidate which parameters govern the transport of pesticides in unamended and amended soils due to the changes in soil heterogeneity after amendment and flow conditions (Kodesova et al., 2012; Marín-Benito et al., 2013, 2015; Thevenot and Dousset, 2015).

Tebuconazole and cymoxanil are two fungicides widely used on vineyards to combat powdery mildew fungi. They are compounds with different chemical structures and properties. Cymoxanil is a synthetic cyanoacetamide oxime compound and it is considered a non-persistent fungicide (PPDB, 2015). Tebuconazole is a synthetic triazole with a high affinity for soil OC (Herrero-Hernández et al., 2011). Both fungicides could reach the soil after their application to plants, and they have been detected in surface and ground waters in areas of intense fungicide use in Spain (Herrero-Hernández et al., 2013; 2016) and other areas of the world (Battaglin et al., 2011; De Geronimo et al., 2014; Montagner et al., 2014).

Immobilization of both fungicides in soil amended with the organic residue spent mushroom substrate (SMS) has been studied (Álvarez-Martín et al., 2016a) using different doses of residues to evaluate effect of SMS applied as organic amendment or as barrier to avoid diffuse or point contamination by the application of these compounds. Immobilization of these compounds may be the first action to avoid water contamination, although the study of other processes such as leaching and/or degradation of these compounds immobilized in amended soils are required to reveal the effect of SMS

on the behavior of fungicides. Dissipation of tebuconazole and cymoxanil in SMS amended soils earlier carried out (Álvarez-Martín et al., 2016b) revealed SMS reduces the extractable fraction of fungicides through the formation of non-extractable residues. Therefore, other processes such as fungicide leaching should be taken into account to have a further view of the effect of the proposed soil amendment strategy.

The aim of this work was to study the mobility of tebuconazole and cymoxanil in a vineyard soil unamended and amended with the organic residue SMS at low rate (5%), simulating its application to soil as an organic amendment, and at high rate (50%), simulating its application to soil as a barrier. Leaching of both fungicides was carried out using packed soil columns under different conditions such as: i) the rate of SMS applied to soil, ii) the flow of water applied (saturated or saturated-non saturated) and iii) the incubation time of the fungicide in the soil (1 and 30 days) in order to assess the influence of these factors on the mobility of tebuconazole and cymoxanil which are widely applied in different types of crops.

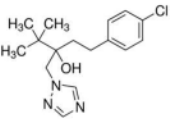
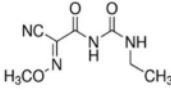
2 MATERIAL AND METHODS

2.1 Chemicals

Fungicides, tebuconazole and cymoxanil, were used as unlabelled and labelled compounds. Unlabelled compounds (purity >98%) were supplied by Sigma-Aldrich Química SA (Madrid, Spain) and labelled compounds were supplied by IZOTOP Co., Ltd., (Budapest, Hungary). [Acetyl-2- ^{14}C]-cymoxanil was with a specific activity of 10.08 MBq/mg and 98.45% purity) and [triazole-U- ^{14}C]-tebuconazole was with a specific activity of 4.72 MBq/mg and 98.06% purity).

Table 1 shows their physicochemical properties and environmental fate parameters (PPDB, 2015). Tebuconazole is classified as non-polar and immobile whilst cymoxanil is done as polar and mobile according to the classification of non-polar when the log Kow value is > 3.0 and as mobile when the log Koc is <2.5 (Delle Site, 2001).

Table 1. Chemical structure and physicochemical properties of fungicides studied.

| Common name IUPAC name | Chemical structure | WS ^a (mg L ⁻¹) | Log Kow ^b | Koc ^c (mL g ⁻¹) | DT ₅₀ (days) | GUS index |
|--|---|---|-------------------------|---|----------------------------|--------------|
| Tebuconazole (<i>RS</i>)-1- <i>p</i> -chlorophenyl-4,4-dimethyl-3-(1 <i>H</i> -1,2,4-triazol-1-ylmethyl)pentan-3-ol |  | 36 | 3.7 | 769 | 63-365 | 2.0 |
| Cymoxanil 1-[(<i>EZ</i>)-2-cyano-2-methoxyiminoacetyl]-3-ethylurea |  | 780 | 0.67 | 43.6 | 0.7-3.5 | 0.3 |

^aWS solubility in water at 20 °C, ^bKow octanol/water partition coefficient at pH 7 and 20°C, ^cKoc sorption coefficient normalized to organic carbon content, DT₅₀ soil degradation (aerobic), GUS leaching potential index. From PPDB, Pesticide Properties DataBase, 2015. (<http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm>).

2.2 Soil and amendment

Spent mushroom substrate (SMS) from *Agaricus bisporus* cultivation was supplied by Sustratos de La Rioja S.L. (Pradejón, Spain). Their physicochemical characteristics determined as described by Marín-Benito et al. (2012) are pH 6.97, ash content 33.6%, OC content 24.5%, DOC 1.91% and moisture content 64.5%.

Soil sample was collected from the surface horizon (0-30 cm) in a vineyard located in Sajazarra (42°35'18"N, 2°57'41"W) in La Rioja region. The soil was air-dried and sieved (<2 mm) to determine its characteristics using standard analytical methods (MAPA, 1986). Soil texture was classified as sandy clay loam (67.0% sand, 11.9% silt, 21.1% clay and 51.0% carbonate content). The soil was amended with SMS at 5% and 50% (w/w) on a dry weight basis. The pH and OC content were 7.52 and 0.67% (unamended soil, S), 7.26 and 1.73% (soil amended with 5% of SMS, S+SMS5), and 7.19 and 16.3% (soil amended with 50% of SMS, S+SMS50), respectively.

2.3 Soil column setup / leaching studies

Leaching experiments were performed in glass columns of 3 cm (i.d) x 25 cm (length) packed with 100 g of soil and soil amended with 5% of SMS or 80g in the case of the soil amended with 50% of SMS. Each column was oversaturated from the bottom with distilled water to their maximal water holding capacity and then left to equilibrate, so the humidity conditions were equivalent to field capacity. The pore volume (PV) of the packed columns was estimated by the difference in weight between water saturated columns and oven dry columns. Pesticides were applied in the top part of the columns by adding 1 mL of a solution of 1 mg mL⁻¹ in methanol with a specific activity of 10 kBq mL⁻¹. The leaching was carried out 24 h after the fungicide application in the soil column (non-incubated) and after one month (incubated at 20°C in the dark). In order to minimize the soil mineral balance disruption, CaCl₂ (0.01M) was used instead water. Two different washing flows were applied, saturated and saturated-non saturated. Under saturated flow conditions, 500mL of CaCl₂ solution was continuously pumped. Under saturated-non saturated flow conditions, the fungicides were leached by adding the same volume of CaCl₂ during 20 days (25 mL per day) up to 500 mL. Mineralization of fungicides over incubation time in the column or over saturated-non saturated flow time was measured. A ¹⁴CO₂ trap, consisting of a scintillation vial containing 1 M NaOH (1 mL) was attached on the top of the column and ¹⁴CO₂ from mineralized ¹⁴C-fungicides was periodically determined.

The flow rate was maintained constant at 1 mL min⁻¹ by a Gilson Miniplus 3 peristaltic pump (Gilson, Inc., Middleton, WI, USA). Fractions of leaching solution (15 mL) were taken by a Gilson F203 automated fraction collector. Each experiment was carried out in duplicate. After the leaching, the columns were cut into three segments (0-5 cm, 5-10 cm and 10-15 cm) and the soil contained in each segment was turned over and weighed.

The tracer chosen was KCl. It is considered to be a conservative tracer since the quantity of KCl injected was fully recovered at the outlet of the column. Chloride ion was applied at the rate of 47 mg per column (1 mL from KCl solution of 100 g L⁻¹ in water). Water flow rates were the same as those used in the fungicide leaching setting (saturated flow and saturated- non saturated flow).

2.4 Pesticide analysis

The ^{14}C -fungicide concentrations in the leached fractions were determined by mixing 1 mL of leachate with 4 mL of scintillation liquid (EcoscintTM A, National Diagnostics, Hesse, Germany), then measuring the activity of the pesticide in disintegration per minute (dpm) on a Beckman LS6500 Liquid Scintillation Counter (Beckman Instruments Inc., Fullerton, CA, USA). The dpm value recorded was related to the dpm obtained for the aliquots of the respective standards of the pesticide solutions. Limit of quantification for ^{14}C -fungicide was determined as the background radioactivity (19-50 dpm) in the CaCl_2 leached. In all solutions, determinations were carried out by duplicate, and the range of the coefficient of variation was always between 0.1 - 2%. $^{14}\text{CO}_2$ from mineralized ^{14}C -fungicides in the scintillation vial containing 1M NaOH (1 mL) was determined by mixing with 4 mL of scintillation cocktail, and determined as previously indicated.

The retained ^{14}C -fungicide remaining in the soil after leaching was determined by soil combustion of triplicate 1 g dried soil samples from each segment of the column using a Biological Oxidizer OX500 (R.J Harvey Instrument Corporation, Tappan, NY, USA) under O_2 excess at 900 °C. The $^{14}\text{CO}_2$ generated was trapped in a mixture of ethanolamine (1 mL) and scintillation cocktail (Oxysolve C-400, Zinsser Analytic, Berkshire, UK, 15 mL), and determined as indicated above.

Chloride ion concentrations were determined using a Metrohm Ion Chromatograph (Metrohm Ltd., Switzerland) with a conductivity detector. The chloride ion concentrations were determined following the method described by Rodríguez-Cruz et al. (2011).

2.5 Data analysis

Retardation factors, R , were determined as indicators of the maximum peak shifts of the breakthrough curve (BTC) for pesticide leaching in unamended and amended soil columns. These factors were calculated according to the expression $R = 1 + K\rho/\theta$ (Kookana and Aylmore, 1993) and assuming that sorption-desorption isotherms are linear and reversible, where ρ is the bulk density of the soil (g cm^{-3}), θ is the volumetric water content or pore volume in the packed column divided by the total volume ($\text{cm}^3 \text{ cm}^{-3}$) and K is the distribution coefficient for linear sorption of fungicides by soil (mL g^{-1}). The parameters of soil columns and the calculated values of sorption constants (K) and retardation factors (R) are included in Table 2 and Table 3 for tebuconazole and cymoxanil, respectively.

Table 2. Distribution coefficients (K_d), parameters of soil columns (pore volume, density and porosity) and retardation factor (R) for tebuconazole in unamended (S) or amended soils (S+SMS5 and S+SMS50)

| Column/Flux | Fungicide treatment | $K_d \pm SD^a$ (mL g ⁻¹) | PV \pm SD (mL) | $\rho \pm SD$ (g cm ⁻³) | $\Theta \pm SD$ (cm ³ cm ⁻³) | $R \pm SD$ |
|-------------------------|---------------------|---|------------------|--|--|-----------------|
| Soil (S) | | 0.11 \pm 0.01 | | | | |
| Saturated | Non incubated | | 42.60 \pm 0.59 | 1.21 \pm 0.02 | 0.52 \pm 0.00 | 4.90 \pm 0.05 |
| Saturated-Non Saturated | Non incubated | | 39.89 \pm 1.14 | 1.20 \pm 0.01 | 0.48 \pm 0.02 | 5.16 \pm 0.12 |
| Saturated | Incubated | | 37.49 \pm 3.28 | 1.18 \pm 0.01 | 0.44 \pm 0.04 | 5.44 \pm 0.39 |
| Saturated-Non Saturated | Incubated | | 38.45 \pm 0.04 | 1.20 \pm 0.01 | 0.46 \pm 0.00 | 5.32 \pm 0.00 |
| S+SMS5 | | 0.33 \pm 0.06 | | | | |
| Saturated | Non incubated | | 44.48 \pm 1.50 | 1.19 \pm 0.02 | 0.53 \pm 0.03 | 10.8 \pm 0.33 |
| Saturated-Non Saturated | Non incubated | | 42.41 \pm 0.25 | 1.17 \pm 0.02 | 0.50 \pm 0.01 | 11.3 \pm 0.06 |
| Saturated | Incubated | | 43.49 \pm 2.64 | 1.18 \pm 0.01 | 0.51 \pm 0.03 | 11.1 \pm 0.61 |
| Saturated-Non Saturated | Incubated | | 43.47 \pm 0 | 1.20 \pm 0.01 | 0.52 \pm 0.01 | 11.1 \pm 0.00 |
| S+SMS50 | | 3.28 \pm 0.06 | | | | |
| Saturated | Non incubated | | 42.95 \pm 1.59 | 0.65 \pm 0.01 | 0.35 \pm 0.02 | 62.2 \pm 2.25 |
| Saturated-Non Saturated | Non incubated | | 46.79 \pm 5.53 | 0.71 \pm 0.1 | 0.42 \pm 0.11 | 57.6 \pm 6.60 |
| Saturated | Incubated | | 46.73 \pm 4.05 | 0.68 \pm 0.08 | 0.40 \pm 0.08 | 57.5 \pm 4.85 |
| Saturated-Non Saturated | Incubated | | 47.94 \pm 4.81 | 0.69 \pm 0.08 | 0.42 \pm 0.09 | 56.1 \pm 5.50 |

^aStandard deviation of replicates.**Table 3.** Distribution coefficients (K_d), parameters of soil columns (pore volume, density and porosity) and retardation factor (R) for cymoxanil in unamended (S) or amended soils (S+SMS5 and S+SMS50)

| Column/Flux | Fungicide treatment | $K_d \pm SD^a$ (mL g ⁻¹) | PV \pm SD (mL) | $\rho \pm SD$ (g cm ⁻³) | $\Theta \pm SD$ (cm ³ cm ⁻³) | $R \pm SD$ |
|-------------------------|---------------------|---|------------------|--|--|-----------------|
| Soil (S) | | 1.67 \pm 0.01 | | | | |
| Saturated | Non incubated | | 42.47 \pm 0.38 | 1.15 \pm 0.02 | 0.48 \pm 0.01 | 1.22 \pm 0.00 |
| Saturated-Non Saturated | Non incubated | | 39.97 \pm 1.19 | 1.15 \pm 0.00 | 0.46 \pm 0.01 | 1.23 \pm 0.01 |
| Saturated | Incubated | | 41.71 \pm 2.14 | 1.23 \pm 0.09 | 0.51 \pm 0.01 | 1.22 \pm 0.03 |
| Saturated-Non Saturated | Incubated | | 42.13 \pm 0.92 | 1.15 \pm 0.00 | 0.48 \pm 0.01 | 1.22 \pm 0.01 |
| S+SMS5 | | 4.38 \pm 0.30 | | | | |
| Saturated | Non incubated | | 44.06 \pm 0.40 | 1.11 \pm 0.01 | 0.49 \pm 0.01 | 1.75 \pm 0.01 |
| Saturated-Non Saturated | Non incubated | | 43.41 \pm 0.41 | 1.13 \pm 0.01 | 0.49 \pm 0.00 | 1.76 \pm 0.01 |
| Saturated | Incubated | | 44.48 \pm 0.59 | 1.12 \pm 0.00 | 0.50 \pm 0.01 | 1.74 \pm 0.02 |
| Saturated-Non Saturated | Incubated | | 44.90 \pm 0.58 | 1.11 \pm 0.01 | 0.50 \pm 0.00 | 1.74 \pm 0.01 |
| S+SMS50 | | 32.8 \pm 0.49 | | | | |
| Saturated | Non incubated | | 38.36 \pm 1.81 | 0.81 \pm 0.03 | 0.31 \pm 0.02 | 9.55 \pm 0.40 |
| Saturated-Non Saturated | Non incubated | | 40.66 \pm 0.41 | 0.88 \pm 0.02 | 0.36 \pm 0.01 | 9.06 \pm 0.08 |
| Saturated | Incubated | | 38.72 \pm 0.26 | 0.84 \pm 0.04 | 0.33 \pm 0.02 | 9.46 \pm 0.06 |
| Saturated-Non Saturated | Incubated | | 42.16 \pm 0.57 | 0.82 \pm 0.01 | 0.35 \pm 0.00 | 8.77 \pm 0.11 |

^aStandard deviation of replicates.

3 RESULTS AND DISCUSSION

3.1 Leaching of chloride ion in unamended and SMS amended soil columns

Chloride ion breakthrough curves (BTCs) were obtained in all soils and for different flow conditions to explain if changes in leaching of both studied fungicides are related to flow conditions. These BTCs of the non-reactive chloride ion were included in Fig. 1 and 2. They were symmetric and presented peak concentrations (maximum concentration of leached ion obtained in the BTCs during the leaching experiment) up to 47% (saturated flow) or up to 77% (saturated-non-saturated flow) of the applied compound in unamended and amended soils. These peaks were close to 1 PV under both flow conditions. This indicates that the tracer ion leaching is not affected by the water flow system carried out. A reduction in peak concentration was observed for SMS amended soils together some broadening of the BTCs relative to the unamended soil, especially when saturated flow was applied. Chloride ions were present in amended soil in amounts ~ 2-10% (S, S+SMS5 and S+SMS50) regards to the amount of chloride ion added and they could have different leaching than ion added. This was also observed by Marín-Benito et al. (2013) in soils amended with different organic residues. Maximum peaks were around 1 PV when was eluted the water volume filling column void indicating uniform water flow and the absence of preferential flux during the elution of water throughout the soil columns and being retardation factor R for ion chloride in all conditions close to 1. It occurs for conservative ions which do not undergo retention or degradation in soils and no interaction of chloride with the soils is produced (Sánchez et al., 2003), according to an amount chloride ion next to 100% was recovered at the end of leaching process.

3.2 Leaching of tebuconazole in unamended and SMS amended soil columns for different conditions applied

Fig. 1 includes experimental BTCs corresponding to the leaching of tebuconazole in unamended soil columns and in columns of soil amended with SMS at two rates for two different flow conditions (saturated and saturated-non saturated) and incubation time of fungicide in soil (one day and 30 days of residence of fungicide in soil previously to leaching). Firstly, it was observed BTCs of fungicide were very different from those of the non-reactive tracer. BTCs obtained in all conditions were asymmetrical, although all of them presented a rapid initial leaching of ^{14}C applied to the column.

In all the systems, a peak of maximum concentration resulted for leaching of tebuconazole in unamended soil for a water volume in the range 1.14-1.41 PV similar to that shown for the conservative chloride ion, although with a lower concentration (Table 4). This maximum peak represents 3.52-4.92% of the total ^{14}C applied to the column. Then BTCs showed a long tail with shoulders (saturated flow) or peaks (saturated-non saturated flow) indicating steady leaching of the fungicide up to 500 mL (~12PV) of CaCl_2 solution was pumped under saturated or saturated-non saturated flow conditions. The asymmetric sharp observed shows a time-dependent interaction between tebuconazole and soil components, and it could come from a non-equilibrium adsorption (Brusseau et al., 1989). Studies with other chemicals have indicated increase of non-equilibrium adsorption effects by organic amendments such as asymmetrical breakthrough curves (with or without tails) on pesticide leaching (Khorram et al., 2015). The BTC pattern was similar for leaching of tebuconazole after 30 days incubation in the soil column, although peaks of

lower concentrations than for non-incubated fungicide (3.41-4.17% of the total ^{14}C applied to the column) were found.

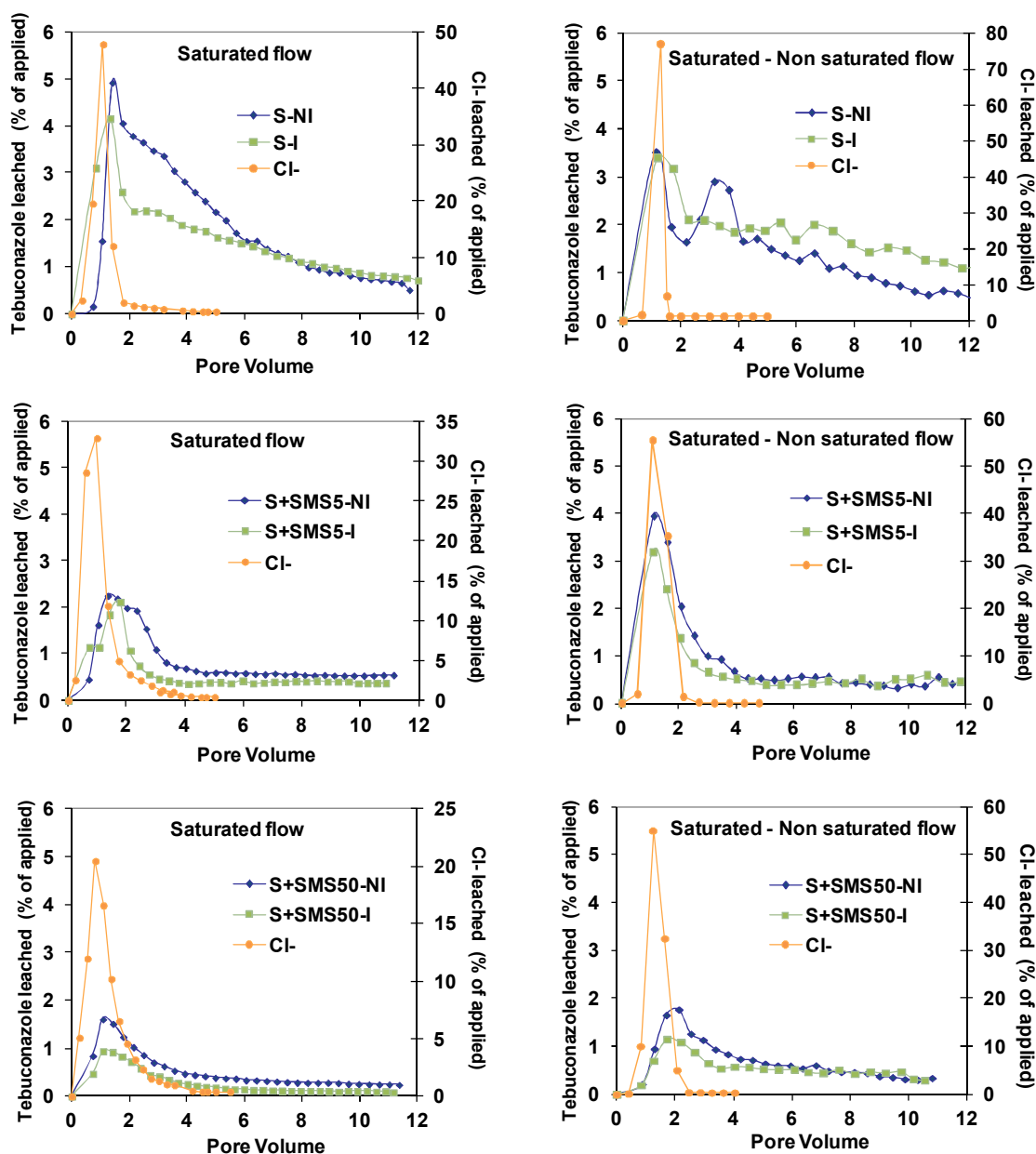


Figure 1. Breakthrough curves for the leaching of conservative chloride ion and tebuconazole without incubation (NI) and after incubation (I) in unamended soil (S) columns and in soil columns amended with spent mushroom substrate at rates of 5% (S+SMS5) and 50% (S+SMS50) when different flows were applied to the columns

In amended soils, BTCs showed also peaks for an initial water volume ranging between 1.06-1.35 PV (S+SMS5) and 1.08-2.15 PV (S+SMS50) (Table 4) similar to indicated for unamended soil and conservative ion and the maximum concentration was in the range 2.25-3.95% (S+SMS5) and 1.61-1.77% (S+SMS50) of the total ^{14}C applied to the column. BTCs patterns were similar for the leaching of tebuconazole non-incubated and after incubation in the soil column, although peak concentrations were decreased after incubation of the fungicide in the column. BTCs obtained indicated peaks of lower concentration in amended soils and less asymmetric curves than in unamended soil, although leaching of ^{14}C was also continuously determined up to 12PV of CaCl_2 solution was pumped under saturated or saturated-non saturated flow conditions. It is noticed that low ^{14}C concentrations were always measured in the leachates.

Table 4. Total amounts of leached and retained tebuconazole (% of applied) for columns of unamended and amended soil, non-incubated and incubated, and under two washing flows.

| Parameters | Non-Incubated soil column | | Incubated soil column | |
|----------------|---------------------------|----------------------------|----------------------------|---------------|
| | Saturated | Sat - Non Sat | Saturated | Sat - Non Sat |
| Soil (S) | | | | |
| Max. Peak | 4.92 ± 0.33 | 3.52 ± 3.57 2.90 ± 0.50 | 4.17 ± 0.57 | 3.41 ± 0.10 |
| PV | 1.41 ± 0.07 | 1.14 ± 0.02 3.17 ± 0.04 | 1.35 ± 0.34 | 1.19 ± 0.04 |
| Total retained | 24.2 ± 0.30 | 50.7 ± 12.4 | 40.2 ± 4.08 | 49.6 ± 13.8 |
| Ttotal leached | 58.7 ± 2.96 | 32.9 ± 13.9 | 46.7 ± 0.92 | 38.4 ± 4.46 |
| Total column | 82.8 ± 3.26 | 83.9 ± 0.98 | 86.9 ± 5.00 | 87.9 ± 9.31 |
| S+SMS5 | | | | |
| Max. Peak | 2.25 ± 0.18 | 3.95 ± 0.02 | 1.15 ± 0.12 2.12 ± 0.83 | 3.19 ± 1.08 |
| PV | 1.35 ± 0.05 | 1.12 ± 0.03 | 1.06 ± 0.01 1.77 ± 0.04 | 1.09 ± 0.01 |
| Total retained | 65.4 ± 4.41 | 77.7 ± 1.58 | 77.9 ± 1.80 | 86.9 ± 5.78 |
| Ttotal leached | 27.4 ± 1.71 | 21.6 ± 3.40 | 18.0 ± 0.67 | 16.8 ± 1.22 |
| Total column | 92.9 ± 2.70 | 99.2 ± 4.90 | 94.9 ± 2.33 | 103 ± 7.00 |
| S+SMS50 | | | | |
| Max. Peak | 1.61 ± 0.18 | 1.77 ± 0.26 | 0.96 ± 0.23 | 1.15 ± 0.10 |
| PV | 1.08 ± 0.24 | 2.15 ± 0.25 | 1.08 ± 0.45 | 1.73 ± 0.19 |
| Total retained | 87.6 ± 1.81 | 88.4 ± 4.37 | 94.9 ± 2.42 | 93.2 ± 2.08 |
| Ttotal leached | 16.5 ± 1.10 | 16.7 ± 0.23 | 9.16 ± 1.04 | 11.8 ± 0.25 |
| Total column | 104 ± 0.70 | 105 ± 4.14 | 104 ± 1.38 | 105 ± 2.33 |

BTCs indicated an apparent slower leaching kinetics of tebuconazole in unamended than in amended soils and this could suggest a stronger interaction of the fungicide with the unamended soil despite adsorption constants were higher in the amended soils than in the unamended one (Table 2). Leaching of a compound non retained by the soil, which was leached in the first PV, did not match with the leaching of tebuconazole according to its reported behavior (EFSA, 2008). To clarify this effect, a leaching experiment in unamended soil using non labeled fungicide and testing its identity by HPLC with mass spectrometry detection as indicated by Herrero-Hernández et al. (2011) was carried out. Results indicated that the initial leached amount did not correspond with ^{14}C -tebuconazole, which represented a low percentage of the total ^{14}C applied to the column, and it could correspond to a possible metabolite of tebuconazole (1,2,4-triazole) highly soluble in water and with a low adsorption coefficient (PPDB, 2015). This metabolite may be present as an impurity of the ^{14}C -labeled fungicide (radiochemical purity of 95.48%). This compound could show a similar sorption behavior as the tebuconazole, being also adsorbed by amended soil, which could explain the decreased peak concentration in S+SMS relative to unamended soil.

Leached and retained amounts of tebuconazole expressed as percentages of the amount initially added to the column (Table 4) agree with the adsorption constants of tebuconazole by soils. Leached amount of tebuconazole after application of 12 PV to the column was in the range (32.9-58.7%) in unamended soil when different conditions were applied. It was higher than those in amended soil (16.8-27.4% (S+SMS5) and 9.16-16.7% (S+SMS50)). In general, these leached amounts were higher when a saturated flow instead of a saturated-non saturated flow was applied to the column and these amounts decreased when the fungicide was incubated in the column. Adsorption increased over time as indicated in a previous study about the dissipation mechanism of this fungicide (Álvarez-Martín et al., 2016b), where it was found that after 1 month incubation period the fraction extracted with a water solution decreased and it was lower in S+SMS50 than in S and S+SMS5. However, the non-extractable fraction of tebuconazole increased over time and it was higher in S+SMS50 than in S and S+SMS5. The increase in this fraction could explain the lower leached amount after incubation. Leached amounts are in agreement with retained amounts in the columns (Table 4), and they were lower in unamended soil (24.2-50.7%) than in amended soils (65.4-86.9% (S+SMS5) and 87.6-94.9% (S+SMS50)). Fig. 2 indicates the distribution of ^{14}C retained in the different segments of the columns as percentage of the total ^{14}C applied under the different conditions studied. Retention of fungicide was mainly in the first segment of the soil column, >50% of amount in the column, under all conditions studied and in S+SMS50, an amount >70% or >80% was found in this first segment when the compound was non-incubated or incubated, respectively. It is noticed that no mineralization of tebuconazole was detected in any of the systems studied being the total ^{14}C recovery >83% (S), >93% (S+SMS5) and >100% (S+SMS50).

Retardation factors, R , were calculated to evaluate the shift of BTC maximum peaks of fungicide leaching in unamended and amended soil columns. These factors which depend on the distribution coefficient (K) are frequently correlated with the PV corresponding to BTC peaks. However R values obtained here (Table 2) are not in agreement with the PV values corresponding to the maximum peaks. R values obtained varied in the ranges 4.90-5.44 (S), 10.8-11.3 (S+SMS5) and 56.1-62.2 (S+SMS50) for fungicide leaching under the different conditions studied. The higher R values obtained in S+SMS

indicate lower mobility of tebuconazole in SMS amended soil owing to the greater adsorption of fungicide by this soil with high OC content. Adsorption of tebuconazole by unamended soil increased 2 times and 13 times when this soil was amended with SMS at 5% and 50%, respectively, due to the increase in soil OC provided by the SMS. In a previous work, a significant correlation between adsorption coefficients and OC contents of SMS amended soils was reported (Álvarez-Martín et al., 2016a). Accordingly, application of SMS to soil at different rates to soil could be used as a tool to enhance the retention of fungicide by amended soil and avoid the presence of leaching peaks as it was found for other pesticides in soils amended with different organic residues (Marín-Benito et al., 2013; Rodríguez-Cruz et al., 2011).

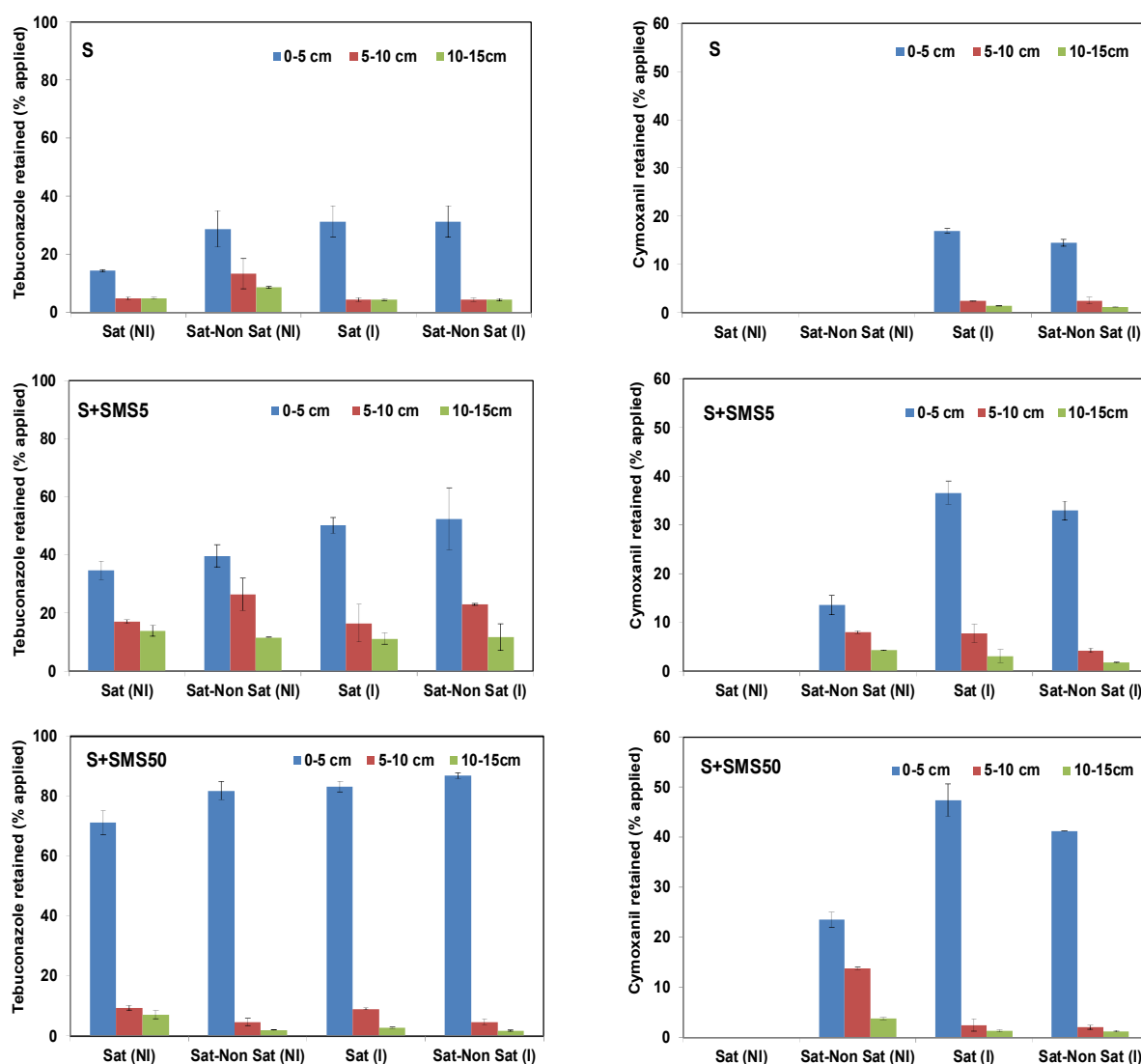


Figure 2. Amounts of tebuconazole (left) and cymoxanil (right) retained in unamended soil (S) columns and in soil columns amended with spent mushroom substrate at rates of 5% (S+SMS5) and 50% (S+SMS50) when different flows were applied to the columns with non incubated and after incubation of fungicides in the columns.

3.3 Leaching of cymoxanil in unamended and SMS amended soil columns for different conditions applied

Fig. 3 includes experimental BTCs corresponding to the leaching of cymoxanil in unamended soil columns and in columns of soil amended with SMS at two rates for two different flow conditions (saturated and saturated-non saturated) and incubation time of fungicide in soil (one day and 30 days of residence of fungicide in soil previously to leaching). BTCs of fungicide leaching were different from those of the non-reactive tracer, although all of them presented a rapid initial leaching of ^{14}C applied to the column. In general, BTCs obtained were asymmetrical, but different patterns were obtained in unamended and amended soils and flow conditions indicating a different behavior of fungicide in the systems studied.

In unamended soil, BTCs of fungicide were symmetric and not very different to those of non-reactive tracer. Maximum peak concentrations were obtained for a PVs close to 1 (range between 0.97-1.56) when leaching was carried out at saturated or saturated-non saturated flow (Table 5), but maximum peak concentrations were different for non-incubated and incubated fungicide in the column. These concentrations represented $\approx 39\%$ of the total ^{14}C applied for non-incubated fungicide in the column, but they decreased up to 12.9-23.1% of the total ^{14}C applied when the fungicide was incubated in the column over 30 days. For both conditions, total leaching of compound was reached at ≈ 3 PVs (Fig. 3). This shape of the curves with short final tails considered similar to that found for the conservative ion would indicate a rapid leaching kinetics of cymoxanil due to weak interaction of the fungicide with the unamended soil.

BTCs obtained in amended soils were less symmetric than those obtained in unamended soil. They presented tails but continuous leaching of ^{14}C was found up to 12 PV of CaCl_2 solution was pumped under saturated or saturated-non saturated flow conditions suggesting different leaching kinetics and a slower leaching process than in the unamended soil. BTCs peaks were obtained for an initial water volume ranging between 1.36-2.84 (Table 5) and the maximum peak concentrations were in the range 13.4-22.3% (S+SMS5) and 8.46-10.8% (S+SMS50) of the total ^{14}C applied to the column for non-incubated compound. The maximum peak concentrations decreased after fungicide incubation in the column (2.42-5.94% (S+SMS5) and 2.89-6.76% (S+SMS50)). These results could be due to the increase in adsorption by the amended soil relative to unamended soil in agreement with the adsorption constants included in Table 3. These constants denote increases of more than 2 and 20 times the adsorption of cymoxanil by S+SMS5 and S+SMS50, respectively (Álvarez-Martín et al., 2016a).

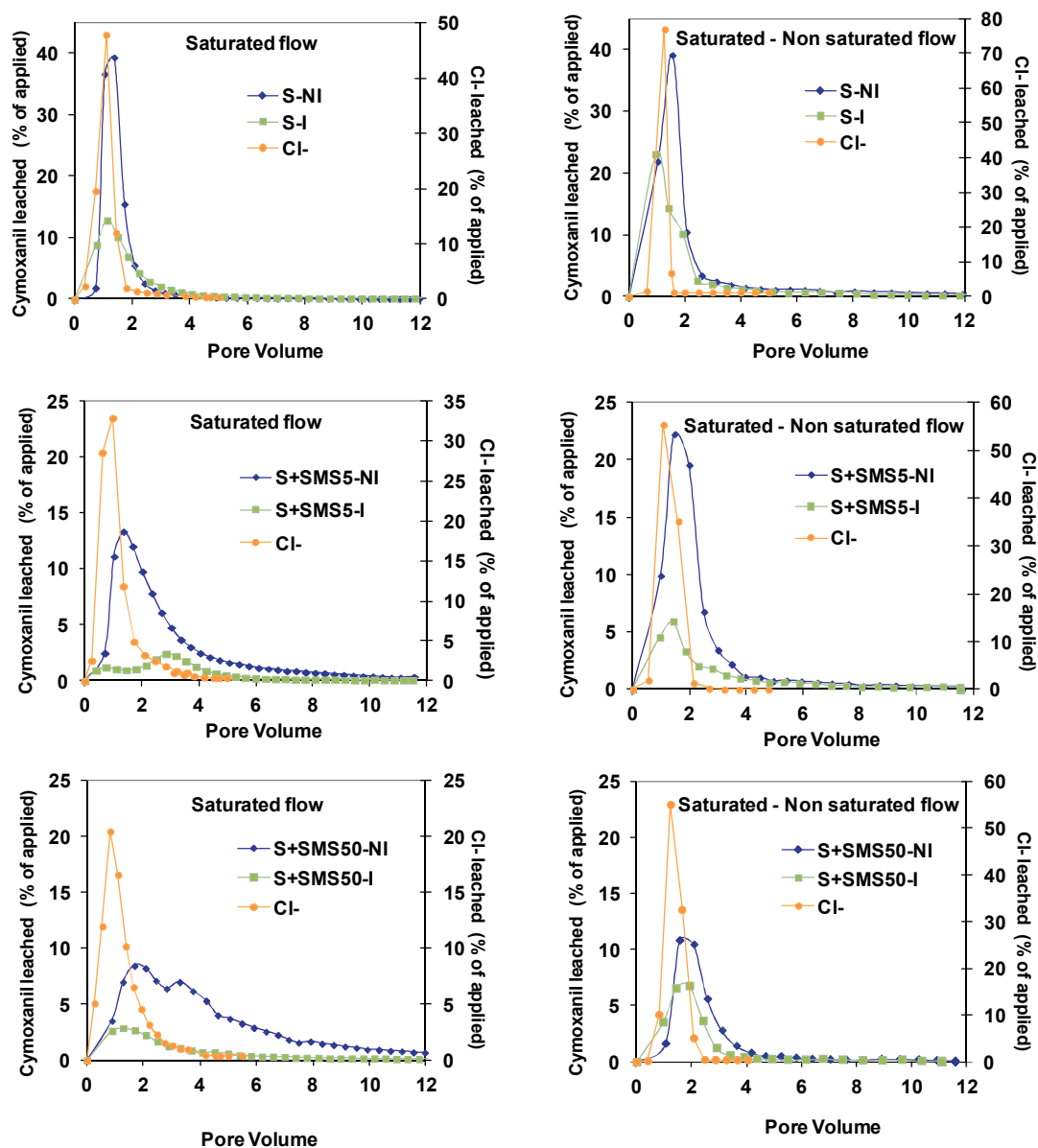


Figure 3. Breakthrough curves for the leaching of conservative chloride ion and cymoxanil without incubation (NI) and after incubation (I) in unamended soil (S) columns and in soil columns amended with spent mushroom substrate at rates of 5% (S+SMS5) and 50% (S+SMS50) when different flows were applied to the columns

The total leached and retained amounts in the columns expressed as percentages of the total ^{14}C amount initially added to the column are included in Table 5. Leaching of non-incubated cymoxanil after application of 12 PV to the column was $\approx 100\%$ in unamended and amended soils when saturated flow was applied indicating no retention of the fungicide in the column under these conditions. Leaching was lower when saturated-non saturated flow was applied to the column (91.9% (S), 72.5% (S+SMS5) and 36.8% (S+SMS50)) and a higher reduction in these amounts was noted when the fungicide was incubated in the column. After incubation, a decreasing in the leached amounts up to 60.5-68.2% (S), 17.2-27.2% (S+SMS5) and 23.4-27.2% (S+SMS50) was noted simultaneously with an increase in the fungicide mineralization. Mineralized amounts were detected up to $\approx 18\%$ (S), $\approx 12\%$ (S+SMS5) and $\approx 10\%$ (S+SMS50) under both applied flows (Table 5). Dissipation mechanism of cymoxanil in SMS amended soil (Álvarez-Martín et al., 2016b) pointed out the mineralization of cymoxanil in unamended and SMS amended soil increased over time reaching an amount $>50\%$ in S and S+SMS5 after 1 month incubation period and $>25\%$ in S+SMS50. These authors reported a decrease of the water extractable fraction over time and they were lower in S+SMS50 than in S and S+SMS5, while the non-extractable fraction of cymoxanil increased over time. These processes could explain the lower leached amount after incubation and suggest that mineralization of cymoxanil may contribute to decrease the leaching of this fungicide when it remains over time in the soil especially with low OM content.

Leached amounts are in agreement with retained amounts in the columns (Table 5). Non-incubated cymoxanil was not retained by the unamended and SMS amended soil when a saturated flow was applied and retained amounts corresponding to 25.9% and 40.9% were found in the first segment of S+SMS5 and S+SMS50 columns when saturated-non saturated flow was applied. After incubation, retention of cymoxanil increased in the S (20.8-18.3%), S+SMS5 (47.5-39.0%) and S+SMS50 (51.0-44.4%) and these amounts were retained mainly in the first segment of the columns. Figure 3 indicates the distribution of ^{14}C in different segments of the columns as percentage of the total ^{14}C applied under different conditions the leaching is studied. Retention of fungicide was found mainly in amended soil according to the increases in adsorption constants of cymoxanil by SMS amended soil regards to unamended one (Álvarez-Martín et al., 2016a).

Retardation factors, R , were calculated to evaluate the shift of BTC maximum peaks of fungicide leaching in unamended and amended soil columns (Tabla 3). R values obtained varied in the ranges 1.22-1.23 (S), 1.74-1.76 (S+SMS5) and 8.77-9.55 (S+SMS50) for fungicide leaching under the different conditions studied. A certain relationship between R values and the PV values corresponding to the maximum peaks was denoted in S and S+SMS5. The higher R values obtained in S+SMS50 indicate lower mobility of cymoxanil owing to the greater adsorption of fungicide by this soil with higher OM content (Álvarez-Martín et al., 2016a). According to this, SMS should facilitate the retention of fungicide by amended soil and may avoid the presence of leaching peaks under controlled conditions while allowing biodegradation of fungicide.

Table 5. Total amounts of leached, retained and mineralized cymoxanil (% of applied) for columns of unamended and amended soil, non-incubated and incubated, and under two washing flows.

| Parameters | Non-Incubated soil column | | Incubated soil column | |
|----------------|---------------------------|---------------|-----------------------|---------------|
| | Saturated | Sat - Non Sat | Saturated | Sat - Non Sat |
| Soil (S) | | | | |
| Max. Peak | 39.5 ± 1.41 | 39.1 ± 4.58 | 12.9 ± 5.93 | 23.1 ± 11.9 |
| PV | 1.38 ± 0.21 | 1.56 ± 0.07 | 1.13 ± 0.13 | 0.97 ± 0.33 |
| Total retained | 0.00 | 0.00 | 20.8 ± 0.46 | 18.3 ± 0.07 |
| Total leached | 101 ± 3.13 | 91.9 ± 1.32 | 60.5 ± 1.11 | 68.2 ± 2.62 |
| Mineralized | 0.21 ± 0.03 | 2.00 ± 0.03 | 18.8 ± 1.93 | 18.6 ± 0.43 |
| Total column | 101 ± 3.16 | 93.9 ± 1.28 | 100 ± 3.04 | 105 ± 2.19 |
| S+SMS5 | | | | |
| Max. Peak | 13.4 ± 2.33 | 22.3 ± 2.48 | 2.42 ± 0.99 | 5.94 ± 2.90 |
| PV | 1.36 ± 0.02 | 1.45 ± 0.38 | 2.84 ± 1.72 | 1.41 ± 0.32 |
| Total retained | 0.00 | 25.9 ± 2.24 | 47.5 ± 6.62 | 39.0 ± 1.59 |
| Total leached | 96.7 ± 3.39 | 72.5 ± 0.94 | 17.2 ± 6.24 | 27.2 ± 2.04 |
| Mineralized | 0.57 ± 0.16 | 5.80 ± 0.39 | 11.6 ± 1.35 | 12.8 ± 2.35 |
| Total column | 97.2 ± 3.23 | 104 ± 2.79 | 76.3 ± 0.98 | 79.0 ± 1.89 |
| S+SMS50 | | | | |
| Max. Peak | 8.46 ± 0.51 | 10.8 ± 1.04 | 2.89 ± 0.06 | 6.76 ± 0.03 |
| | 7.01 ± 0.21 | | | |
| PV | 1.68 ± 0.22 | 1.57 ± 0.40 | 1.28 ± 0.28 | 1.93 ± 0.37 |
| | 3.28 ± 0.31 | | | |
| Total retained | 0.00 | 40.9 ± 1.00 | 51.0 ± 1.78 | 44.4 ± 0.61 |
| Total leached | 98.1 ± 2.98 | 36.8 ± 1.19 | 23.4 ± 0.90 | 27.2 ± 2.04 |
| Mineralized | 0.16 ± 0.00 | 9.38 ± 3.29 | 7.63 ± 1.10 | 10.5 ± 1.52 |
| Total column | 98.3 ± 2.99 | 87.2 ± 4.48 | 82.1 ± 1.98 | 82.1 ± 4.18 |

4 CONCLUSIONS

Results indicated different effects of SMS applied to soil for the leaching of the fungicides tebuconazole and cymoxanil with different characteristics. Leached amounts of tebuconazole in unamended soil decreased up to 2 or 3 times in S+SMS5 and S+SMS50, respectively, when a water volume corresponding to 12 PV was applied as saturated or saturated-non saturated flow. The decrease was greater (2.6 and 5 times in S+SMS5 and S+SMS50, respectively) when leaching experiment was conducted after 30 days incubation of the fungicide in the soil column. Up to more than 65% or 85% could be retained in the SMS amended soil depending on the environmental conditions occurring. Leaching of cymoxanil was more rapid than that of tebuconazole from unamended and SMS amended soils. Total leaching was found in unamended and SMS amended soils under saturated flow condition.

However, a decreasing of 1.3 and 2.6 times in the leaching of fungicide occurred in S+SMS5 and S+SMS50, respectively, when the saturated-non saturated flow was applied. Leached amounts decreased and retained amounts increased similarly in amended soils at the two SMS rates applied after incubation of the fungicide in the soil column relative to unamended soil, although mineralization of the fungicide was found in this soil. In conclusion SMS contributed i) to decrease leaching of tebuconazole increasing the adsorbed amount in a non-extractable form and ii) to decrease leaching of cymoxanil increasing its adsorption and decreasing its mineralization. Both effects should be taken into account when SMS is considered to be used as a tool to prevent water contamination by pesticides with different characteristics.

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Changes in activity and structure of the soil microbial community after application of azoxystrobin or pirimicarb and an organic amendment to an agricultural soil

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Abstract

Nowadays, pesticides and organic amendments are widely used in agricultural soils, the former to improve crop yield and the latter to maintain soil quality and to mitigate pesticide leaching. Both amendments and pesticides can affect soil microbial communities, which may impact crop fitness. The current study investigated the changes that occur to soil microbial communities following the use of spent mushroom substrate (SMS) (2% and 5% on a dry weight basis) added as an amendment to an agricultural soil in combination with the widely used pesticides azoxystrobin or pirimicarb (at 2 and 25 mg kg⁻¹ rates). DT₅₀ values of azoxystrobin (2 mg kg⁻¹) and pirimicarb (2 and 25 mg kg⁻¹) decreased, while DT₅₀ values of azoxystrobin (25 mg kg⁻¹) increased when SMS was applied to soil. Dissipation experiments found that azoxystrobin was more persistent than pirimicarb over the incubation time (90 days). Broad-scale methods such as soil dehydrogenase activity (DHA) and soil respiration showed that SMS rate, pesticide dose and incubation time all affected microbial community metabolism. DHA increased in SMS-amended soils and increased (azoxystrobin) or decreased (pirimicarb) over incubation time. For both pesticides, soil respiration was not stimulated by SMS application to soil, increased in the soils when the concentration of the pesticide increased and decreased with increasing incubation time. Illumina MiSeq analysis of 18S rRNA genes demonstrated a strong effect of the SMS and incubation time, but not the pesticides, on eukaryote community composition.

Keywords: Pesticides, spent mushroom substrate, dissipation, soil microbial community, next generation sequencing, microbial parameters.

1 INTRODUCTION

Pesticides used in agriculture may reach surface and ground waters through non-point and point pollution sources by direct run-off and leaching, or following washing and disposal of empty containers post application (Carter, 2000, Herrero-Hernández et al., 2013). The pollution of surface and ground waters by pesticides is governed by the physicochemical characteristics of the compounds, and soil and environmental variables, which together govern their behaviour (e.g. sorption and degradation) in the environment (Arias-Estévez et al., 2008).

Recently, the use of organic amendments in agricultural soils has been demonstrated to be a feasible way to prevent soil and water pollution by pesticides, because the organic matter (OM) incorporated into the soil with the amendment can immobilize and/or enhance degradation of the pollutant and its metabolites and therefore avoid their dispersion to the environment (Rodríguez-Liebana

et al., 2014). At the same time such additions of residue to soil can improve soil quality and plant productivity (Cotxarrera et al., 2012). Furthermore, use of organic substrates such as soil amendments helps to solve environmental problems associated with the disposal of organic materials into landfill (Fait et al., 2007).

Common amendments with potential value for application to agricultural soils include biochar, sewage sludge, vinasse and spent mushroom substrate (SMS) (Pérez-Piqueres et al., 2006; Castillo et al., 2008). SMS is an organic amendment applied usually to agricultural soils of some areas from Spain where mushrooms are grown in large amounts (www.magrama.gob.es), and it shows potential to sorb hydrophobic pesticides due to its high OM content. Recently, the effect of SMS as a soil amendment to promote sorption or immobilization and degradation of pesticides, and to reduce their mobility in the environment, has been studied in detail (Marín-Benito et al., 2012a, 2012b, 2012c). Results of these studies have identified that organic carbon (OC) and dissolved organic carbon (DOC) of SMS determine its capacity as a sorbent of pesticides and thereby their bioavailability to soil microorganisms and their degradation rates and retention in soil.

However the addition of both pesticides and organic amendments to agricultural soils can affect microbial communities, which play key roles in the delivery of soil ecosystem services, including pollutant biodegradation, biogeochemical cycling and soil formation (Gianfreda and Rao, 2008). In general the biodegradation of pesticides applied to crops can be altered by addition of organic amendments to the soil via i) increasing sorption and reducing bioavailability to microorganisms, ii) introducing exogenous microorganisms with biodegradation abilities or iii) changing soil properties (eg, pH and available nutrients), thereby altering the composition and activity of indigenous microflora, including degrader communities (Arias-Estévez et al., 2008; Cabrera et al., 2014).

In the case of SMS, previous reports have shown that the addition of this amendment to soil stimulates the soil microbial biomass and the activities of the soil microbial community (Pérez-Piqueres et al., 2006; García-Delgado et al., 2015) and can inhibit or stimulate degradation of pesticides depending on the nature and stabilization of SMS and pesticide properties (Herrero-Hernández et al., 2011; Marín-Benito et al. 2012b; Marín-Benito et al., 2014). However, the relative impacts and interactions of SMS and pesticides on soil microbial communities, associated with these effects, are unclear.

The aim of the current work was to study the changes that occur in soil microbial communities due to the combined use of the pesticides azoxystrobin or pirimicarb and SMS as an organic amendment to an agricultural soil from La Rioja region (Spain). Azoxystrobin is a fungicide with a broad spectrum of systemic activity and belonging to the strobilurin group. Pirimicarb is an insecticide with systemic activity in the carbamate group. Both compounds are used in a broad range of crops for the control of fungal and aphid crop pathogens. These two pesticides were applied to an unamended and SMS-amended soil at two rates for characterisation of: 1) Dissipation kinetics of pesticides following extraction with an organic solvent to measure total pesticide concentrations; 2) Soil dehydrogenase activity (DHA) and soil respiration to give an indication of broad-scale impacts of the pesticides and SMS amendment on the soil microbial community, and 3) Illumina MiSeq sequencing of eukaryote 18S rRNA genes, to investigate effects of the pesticides and SMS on the structure and diversity of microbial eukaryote communities,

which are key contributors to soil biological quality, and potentially impacted by fungicide and insecticide applications to soil (Bending et al., 2007).

2 MATERIAL AND METHODS

2.1 Chemicals

Pirimicab [2-dimethylamino-5,6-dimethylpirimidin-4-yl dimethylcarbamate] is a carbamate insecticide. Pirimicarb (water solubility 3100 mg L⁻¹, log K_{ow} 1.7) acts by disabling the nervous system by the inhibition of acetylcholinesterase. Azoxystrobin [methyl (E)-2-{2-[6-(2-cyanophenoxy)pirimidin-4-yloxy]phenyl}-3-methoxyacrylate] is a strobilurin fungicide. Azoxystrobin (water solubility 6.7 mg L⁻¹, log K_{ow} 2.5) is the heme-containing component of the cytochrome bc₁ complex, which accepts electrons from Rieske protein and transfer electrons to cytochrome c in the mitochondrial respiratory chain (Berman et al., 2000). Both pesticides were supplied by Sigma-Aldrich Química SA (Madrid, Spain). All organic solvents and other reagents used were of analytical grade and were supplied by Fisher Scientific Ltd. (Loughborough, UK) and Sigma-Aldrich Química SA (Madrid, Spain).

2.2 Amendment and soil characterization

Spent mushroom substrate (SMS) from *Agaricus bisporus* cultivation is a pasteurized mixture of cereal straw and poultry litter, urea, and gypsum. This residue was supplied by Sustratos de La Rioja SL (Pradejón, Spain). SMS was sieved <2 mm prior to use.

Characteristics of SMS were determined in air dried samples. The pH was determined in a residue/water suspension (1/2.5 w/v ratio). Organic carbon (OC) content was determined by oxidation (Walkley–Black method) (Jackson, 1964). Dissolved organic carbon (DOC) was determined in a suspension of residue in Milli-Q ultrapure water (1/100 w/v ratio) as described by Marín-Benito et al. 2012b. Total N content was determined according to the Kjeldahl method (Bremner, 1996). The organic amendment applied showed the following characteristics (on a percentage dry weight basis): pH 6.97, ash content 33.6%, OC content 24.5%, DOC 1.91%, N content 1.75% and C/N 14.0. Moisture content was 64.5%.

The sandy-loam soil used in all experiments was taken from the surface horizon (0–30 cm) of a vineyard farm in Alcanadre (42°24'20"N, 2°7'6"W, La Rioja, NE-Spain). The soil was sieved (<2 mm) prior to use. Soil characteristics were determined using standard analytical methods (MAPA, 1986). The soil pH was 7.49. The OC content was 0.89%. The particle size distribution determined using the pipette method was 79.6%, 9.0%, 11.4% sand, silt and clay respectively. Inorganic carbon content determined as CaCO₃ with a Bernard calcimeter was 24.1%.

2.3 Experimental design and incubation

The amended soils were prepared by uniformly mixing soil with SMS at a rate of 2% and 5% on a dry weight basis. For soil amended with 2% SMS, the pH was 7.39 and the OC was 1.22, while for soil with 5% SMS, pH and OC were 7.26 and 2.22.

Prior to the addition of pesticides, the soils were incubated in the dark at 20°C for 24 h to equilibrate. The pesticides were dissolved in acetone and added to soil gradually during a 10 min mixing

period to allow the acetone to volatilise. An equal volume of acetone was added to the control samples (i.e. soil and SMS treatments without pesticide). The pesticide concentrations used represented the maximum agronomic dose and 12.5 times the agronomic dose (2 mg kg^{-1} and 25 mg kg^{-1}) considering a soil depth of 5 cm and a bulk density of 1.5 g cc^{-1} . The highest dose of azoxystrobin was similar to that used in previous work, which was shown to alter soil fungal community structure (Bending et al., 2007; Sopeña and Bending, 2013). The same high dose of pirimicarb was used for comparison reasons. Pesticides were studied separately to avoid synergistic effects. Soil water capacity was adjusted to 40%. After mixing thoroughly using sterilized spatulas, 50 g was placed in sterilized bottles and incubated in darkness at 20°C . Soil moisture content was maintained by the addition of sterile distilled water as necessary. A total of 162 bottles were incubated and used in this study reflecting the 3 treatments (unamended soil, and soil mixed with 2 and 5 % SMS) using 3 pesticide application doses (0, 2 and 25 mg kg^{-1}), 2 pesticides, 3 harvest intervals, and 3 replicates.

2.4 Pesticide dissipation

To determine how pesticide persistence in soil was affected by SMS, methanol extraction was carried out. Samples of incubated soil (5 g) were shaken with 10 mL of methanol for 24 h and the extracted pesticide analysed by HPLC/MS using a Waters (Milford, MA, USA) system. Half-life (DT_{50}) was calculated according to FOCUS guidelines (FOCUS, 2006).

HPLC/MS conditions were set up with a mobile phase of acetonitrile:water + 0.1% formic acid, 90:10 and 80:20 (v/v) for pirimicarb and azoxystrobin, respectively. Flow rate was 0.4 ml min^{-1} and retention time was 6.3 min for both pesticides. The studied ion (m/z) $[M]^+$ was 404.0 for azoxystrobin and 239.4 for pirimicarb.

2.5 Dehydrogenase activity and respiration in soils

Soil dehydrogenase activity (DHA) and respiration analysis were performed to give an indication of broad-scale impacts of the SMS and pesticides. DHA was determined following the Tabatabai method (Tabatabai, 1994). The method is based on the extraction and colorimetric determination of the intensely coloured TPF produced from the reduction of colourless TTC in soils. Basal respiration was determined by measuring O_2 absorption by microorganisms in 50 g portions of soil incubated at 25°C for 60 h using Oxi-Top Control equipment (WTW, Weilheim, Germany) according to DIN ISO 16072.

2.6 Microbial community structure analysis

DNA was extracted from all unamended and amended soil samples (0.5 g) using the Fast DNA® Spin Kit for Soil (MP Biomedicals, LLC, UK) according to the manufacturer's instructions. Quality and quantity of the extracted DNA was elucidated by Qubit® BR DNA kit (Life Technologies Corporation). The Q5® Hot Start High-Fidelity PCR Kit (New England Biolabs® inc.) was used to amplify 18S rRNA genes using the primers 18S Euk_1391f/ 18S EukBr (Amaral-Zettler et al., 2009). PCR products were purified using the AMPure XP beads (Beckman Coulter, USA) using the manufacturer's handbook. Index PCR was carried out with the commercial i5 and i7 indexed primers provided by the Illumina Nextera® Index Kit. These PCR products were also purified as mentioned above and DNA concentration was

checked using Quibit. The DNA concentration was normalized to 10 nM and the samples were sent to TGAC (The Genome Analysis Centre, Norwich, UK) for Miseq v2 2 x 250 bp sequencing.

2.7 Processing of Illumina MiSeq Sequence Data

A custom java program was used to attempt to overlap the forward and reverse reads to produce contigs. If there were more than 3 mismatches the reads were discarded, which acted as a quality filtering step. In the case of a mismatch, the base with the higher quality value was inserted into the contig. The resulting contigs were assigned to Operational Taxonomic Units (OTUs) using the UPARSE pipeline (Edgar, 2013). First the sequences were de-replicated using the custom java program and then sorted and any singletons were discarded. Sequences were then clustered at the default 97% identity level and chimeras were removed. Next, de-replicated reads were mapped against OTU sequences, in order to calculate the abundance of each OTU. A custom java program was used to assign the original reads (before de-replication) to each OTU and correlate them to each sample. The OTU sequences were assigned taxonomic classification using QIIME (Caporaso et al. 2010), which implements the RDP classifier (Wang et al., 2007). An OTU table consisting of the OTU counts for each sample and the taxonomic classification was constructed. Once OTUs were assigned a taxonomy, all OTUs annotated as bacteria or unassigned were removed from the OTU table resulting in a set of eukaryote OTUs. These were then rarefied to 4000 and 8000 reads per sample for azoxystrobin and pirimicarb respectively, which was the minimum number of usable reads attained across the samples.

2.8 Statistical analyses

One-way and two-way analysis of variance (ANOVA) were used to evaluate the effects of the different treatments (pesticide concentration, type of soil sample and incubation time) as independent factors on the DHA and respiration, and to investigate any interaction between them. Standard deviation (SD) was used to indicate variability among replicates and Fisher's post-hoc test at a confidence level of 95 % was used to establish significant differences between values. SPSS Statistics 22.0 software for windows (SPSS Inc. Chicago, USA) was used.

Rarefaction Curves were used to determine sequencing sampling depth (Chao, 1984). Significant differences in eukaryote community structure between treatments over the course of the experiment were analysed using Bray-Curtis resemblance matrices using non-metric multidimensional scaling (NMDS) in the program PRIMER Version 6.1.9 (PRIMER-E Ltd., Plymouth, UK). ANOSIM reports the level of dissimilarity between sample groups (global R) and the associated level of significance (P). R is scaled to be within the range +1 to -1. Positive R values indicate that samples are more dissimilar between groups than within groups. R values close to zero occur if the high and low similarities are perfectly mixed and bear no relationship to the group. Negative R values indicate that dissimilarities within groups are greater than dissimilarities between groups (Clarke, 1993). Additionally, similarity percentage (SIMPER) analysis was used to elucidate which specific OTUs contributed to the similarity matrix structure between treatments (Clarke and Warwick, 2001).

3 RESULTS

3.1 Dissipation of azoxystrobin and pirimicarb in unamended and amended soil

Pesticide dissipation followed single first order kinetics with $r^2 > 0.91$ for azoxystrobin and $r^2 > 0.98$ for pirimicarb. The DT_{50} values ranged between 122.5 and 268.2 days for azoxystrobin and 20.0 and 42.0 days for pirimicarb depending on the soil treatment and pesticide dose (Table 1).

DT_{50} values of azoxystrobin decreased (low dose) or increased (high dose) when applied to SMS amended soil ($p < 0.05$), with no significant effect of SMS amendment rate. In all cases, degradation rate of azoxystrobin was slower (4-12 times) than that of pirimicarb. SMS application at 5 %, but not 2 % reduced DT_{50} values of pirimicarb ($p < 0.05$).

Table 1. Dissipation rate (k) and half-live (DT_{50}) values for azoxystrobin and pirimicarb applied at two doses (2 and 25 mg kg⁻¹) in unamended and SMS-amended soils.

| Soils | k (days ⁻¹) | $DT_{50} \pm SD^a$ (days) | r^2 | k (days ⁻¹) | $DT_{50} \pm SD^a$ (days) | r^2 |
|-----------|---------------------------------------|------------------------------|-------|--|---------------------------|-------|
| | Azoxystrobin (2 mg kg ⁻¹) | | | Azoxystrobin (25 mg kg ⁻¹) | | |
| S | 0.004 | 178.8 \pm 15.6 | 0.98 | 0.004 | 190.1 \pm 40.4 | 0.94 |
| S+2%SMS | 0.006 | 122.5 \pm 10.2 | 0.99 | 0.003 | 240.6 \pm 29.9 | 0.91 |
| S+5%SMS | 0.005 | 139.4 \pm 15.0 | 0.99 | 0.003 | 268.2 \pm 27.6 | 0.97 |
| LSD (95%) | 0.001 | 27.59 | | 0.001 | 66.22 | |
| | Pirimicarb (2 mg kg ⁻¹) | | | Pirimicarb (25 mg kg ⁻¹) | | |
| S | 0.018 | 39.0 \pm 7.5 | 0.99 | 0.016 | 42.6 \pm 7.5 | 0.99 |
| S+2%SMS | 0.022 | 31.1 \pm 2.4 | 0.99 | 0.019 | 36.0 \pm 3.0 | 0.99 |
| S+5%SMS | 0.026 | 26.2 \pm 1.7 | 0.99 | 0.035 | 20.0 \pm 0.6 | 0.98 |
| LSD (95%) | 0.005 | 9.266 | | 0.004 | 9.394 | |

^aSD, standard deviation (n=3).

3.2 Dehydrogenase activity in soil

Soil dehydrogenase activity (DHA) was measured to give an indication of broad-scale impacts of the pesticides and SMS on the microbial community and results are included in Fig. 1. In both azoxystrobin and pirimicarb treated soils, DHA activity increased significantly ($p < 0.001$) following SMS application, with the increase greater following 5 % relative to 2 % addition. Furthermore, DHA increased with incubation time ($p < 0.001$) in all treatments (Fig. 1). However, DHA activity was not significantly affected by the concentration of fungicide applied (0, 2 and 25 mg kg⁻¹) in unamended or amended soils. The results of the two-way analysis of variance for DHA in the soils indicated that interaction of time and soil sample variables was significant ($p < 0.01$), not between time and fungicide concentration or soil sample and fungicide concentration.

In the case of pirimicarb, a significant decrease in DHA was seen as the concentration of the insecticide increased ($p < 0.05$) and with increasing incubation time ($p < 0.001$). There was a significant interaction ($p < 0.05$) between soil sample and insecticide concentration variables, but not between time and soil sample ($p > 0.10$) or time and insecticide concentration ($p = 0.10$).

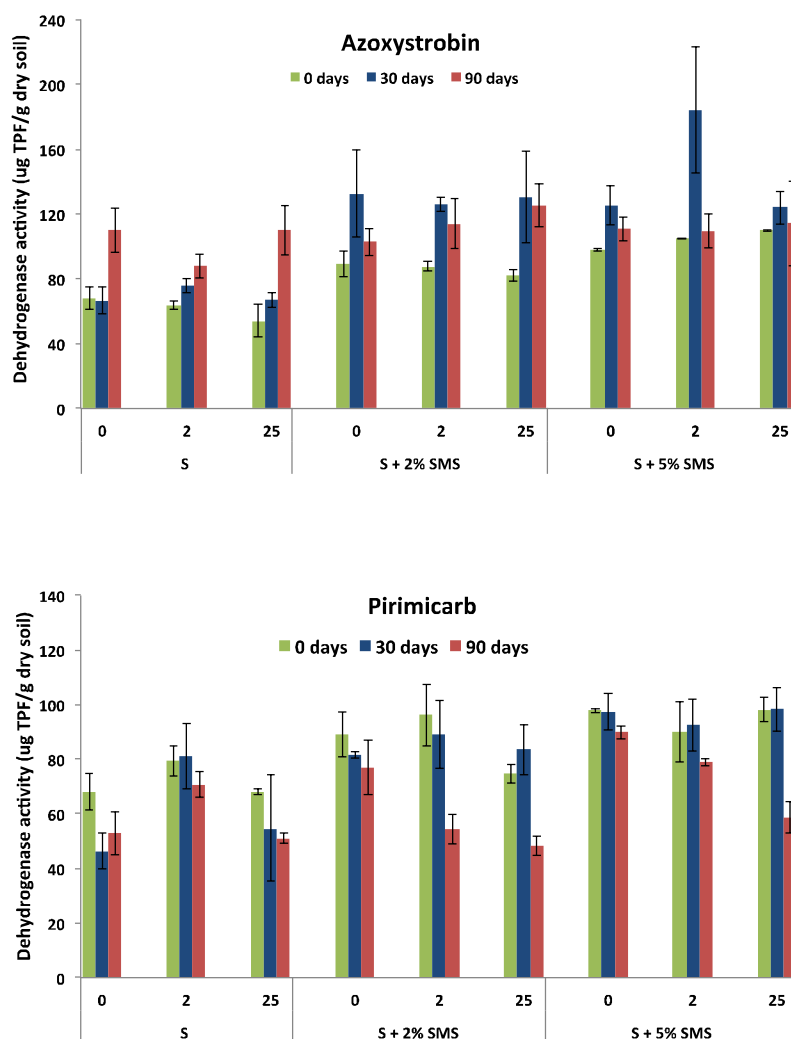


Fig. 1. Soil dehydrogenase activity for unamended and SMS-amended soils, untreated and treated with 2 and 25 mg kg⁻¹ of azoxystrobin or pirimicarb after 0, 30 and 90 days of incubation. Bars indicate the standard deviation of the mean (n=3).

3.3 Soil respiration

In the case of azoxystrobin, SMS application had no significant effect on respiration (Fig. 2). At time 0, respiration was significantly ($p < 0.05$) affected by the concentration of fungicide applied, with a dose dependent increase seen in all treatments. Respiration significantly decreased with incubation time ($p < 0.005$) in all samples (Fig. 2). The results of the two-way analysis of variance for respiration in the soils indicated that interaction of time and fungicide concentration variables was significant ($p < 0.05$), but not between soil amendment and time or pesticide concentration.

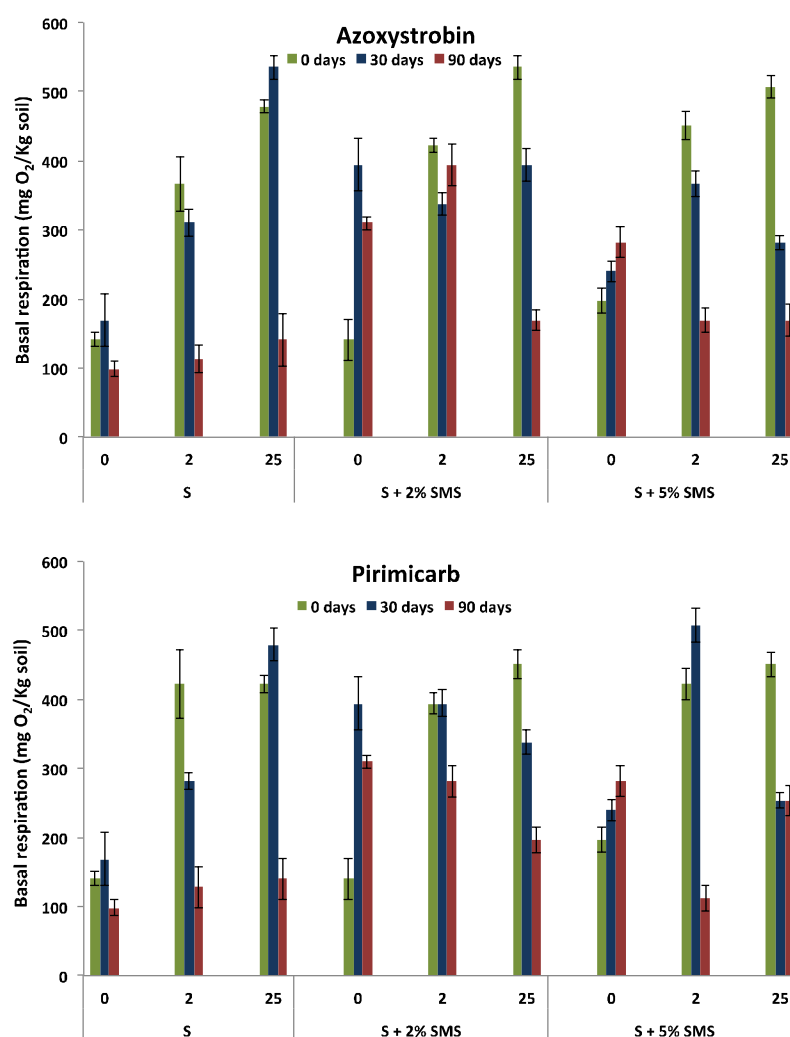


Fig. 2. Soil basal respiration for unamended and SMS-amended soils, untreated and treated with 2 and 25 mg kg⁻¹ of azoxystrobin or pirimicarb after 0, 30 and 90 days of incubation. Bars indicate the standard deviation of the mean (n=3).

For pirimicarb treated soils, SMS application had no significant effect on respiration (Fig. 2). At time 0 there was a significant increase in respiration across all treatments when the concentration of the insecticide increased ($p < 0.05$), although respiration subsequently significantly decreased with increasing incubation time ($p < 0.05$) (Fig. 2). Furthermore a significant interaction ($p < 0.05$) between the time and insecticide concentration variables was observed but not between soil amendment and time or pesticide concentration.

3.4 Soil eukaryote community analysis

Illumina sequencing of eukaryote communities in pirimicarb treated soils (8549617 processed reads) revealed 6293 OTUs with average length of 112 bp, and an average 105551 reads per sample. Among those 1185 OTUs were assigned to fungi.

In the case of azoxystrobin treated soils (8438383 processed reads) there were 5119 eukaryote OTUs with average length of 109 bp, and an average of 104177 sequences per sample. 943 OTUs were assigned to fungi.

Chao1 analysis of the soils treated with pirimicarb (Fig. 3a), showed that there were significant differences (both $p \leq 0.003$) between the unamended soil and SMS amended soils, with amended soils showing 19% higher richness. SMS amendment rate, time and pesticide concentration had no effect on OTU richness (data not shown). Similar results were found for azoxystrobin (Fig. 3b), with significant differences in OTU richness between unamended soil and soil receiving SMS at 2 % and 5% rates (both showed an increase of 12% richness in presence of amendment) ($p \leq 0.003$ and $p \leq 0.015$ respectively).

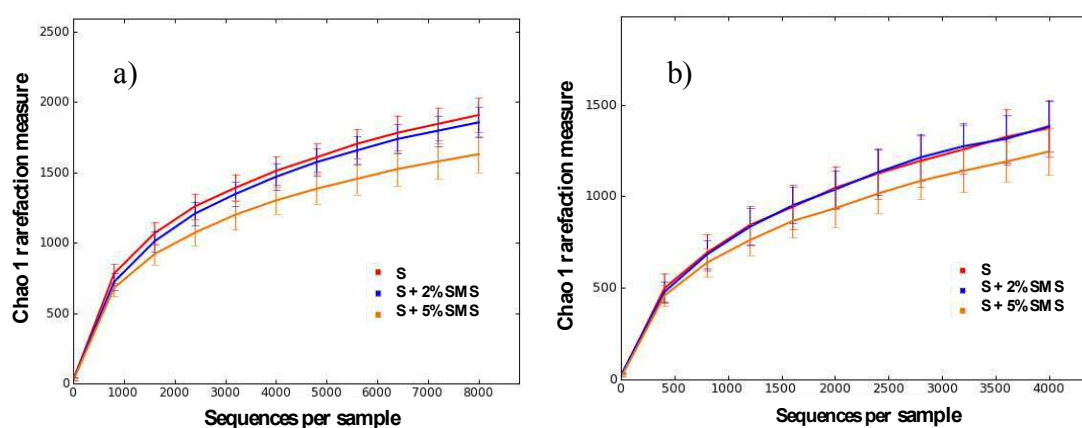


Fig. 3. A diversity estimate Chao1. Eukaryote diversity at the three different soils treated with pirimicarb (a) and azoxystrobin (b). Error bars are ± 0.1 S.E.

NMDS and ANOSIM analyses showed that eukaryote communities in each soil treatment were significantly different ($p < 0.001$) within both azoxystrobin and pirimicarb treated soils (Fig. 4, Table 2). Additionally, within each type of soil (0% SMS, 2% and 5% of SMS) a significant variation was found with harvest time (0, 30 and 90 days) (Table S1).

Table 2. Analyses of similarity (ANOSIM) evaluating the variation of eukaryote communities. (Comparison of percentage of amendment)

| Soils comparisons | Azoxystrobin | | Pirimicarb | |
|-------------------|--------------|-------|------------|-------|
| | R | p | R | p |
| S, S+2%SMS | 0.315 | 0.001 | 0.493 | 0.001 |
| S, S+5%SMS | 0.790 | 0.001 | 0.934 | 0.001 |
| S+2%SMS, S+5%SMS | 0.354 | 0.001 | 0.577 | 0.001 |

However pesticide application had no significant effect on community structure. Furthermore, specific analysis of sequences assigned to fungi, using NMDS and ANOSIM showed similar pattern of variations to the eukaryote analysis (Fig. S1, Tables S2 and S3) with soil treatment and time, but not pesticide application, significantly affecting community structure. SMS addition was associated with increases in the relative abundance of sequences assigned to the Stramenopiles (7-39%), Rhizaria (49-119%) and Heterolobosea (529-1171%), and decreased abundance of sequences assigned to Viridiplantae (27-51%) and Fungi (23-27%) (Fig. 5).

SIMPER analysis was used to identify taxa responsible for differentiating soil treatments. For both azoxystrobin and pirimicarb treated soils, the main taxa responsible for the differences in the eukaryote community across treatments were OTU assigned to the arthropod genera *Symphyleona*, protists related to *Hartmannellidae* sp. and *Stachyamoeba*, and plant sequences related to *Moniliformopses* (Table 3, Table 4). In all cases but for *Stachyamoeba* the relative abundance was decreased in the presence of the amendment.

Different fungal taxa were responsible for differentiating soil treatments in azoxystrobin and pirimicarb amended soils. In the case of azoxystrobin, the major changes were associated to *Agaricomycetes*, *Eurotiomycetes* and *Saccharomycetes* (Table S6). In the case of pirimicarb, we found *Eurotiomycetes*, *Saccharomycetes* and a *Chyridiomycete* (Fig S7). In the SIMPER analysis for analysis of taxa contributing to changes in community composition across harvest times, we found that the main eukaryotic taxa responsible for the differences in the case of azoxystrobin and pirimicarb were *Symphyleona*, *Moniliformopses* and *Hartmannellidae* (Tables S4 and S5). Looking only at fungal structure, we found the taxa most responsible for the observed changes in both pesticides to be *Agaricomycetes*, *Eurotiomycetes*, *Dothideomycetes* and *Saccharomycetes* (Tables S8 and S9).

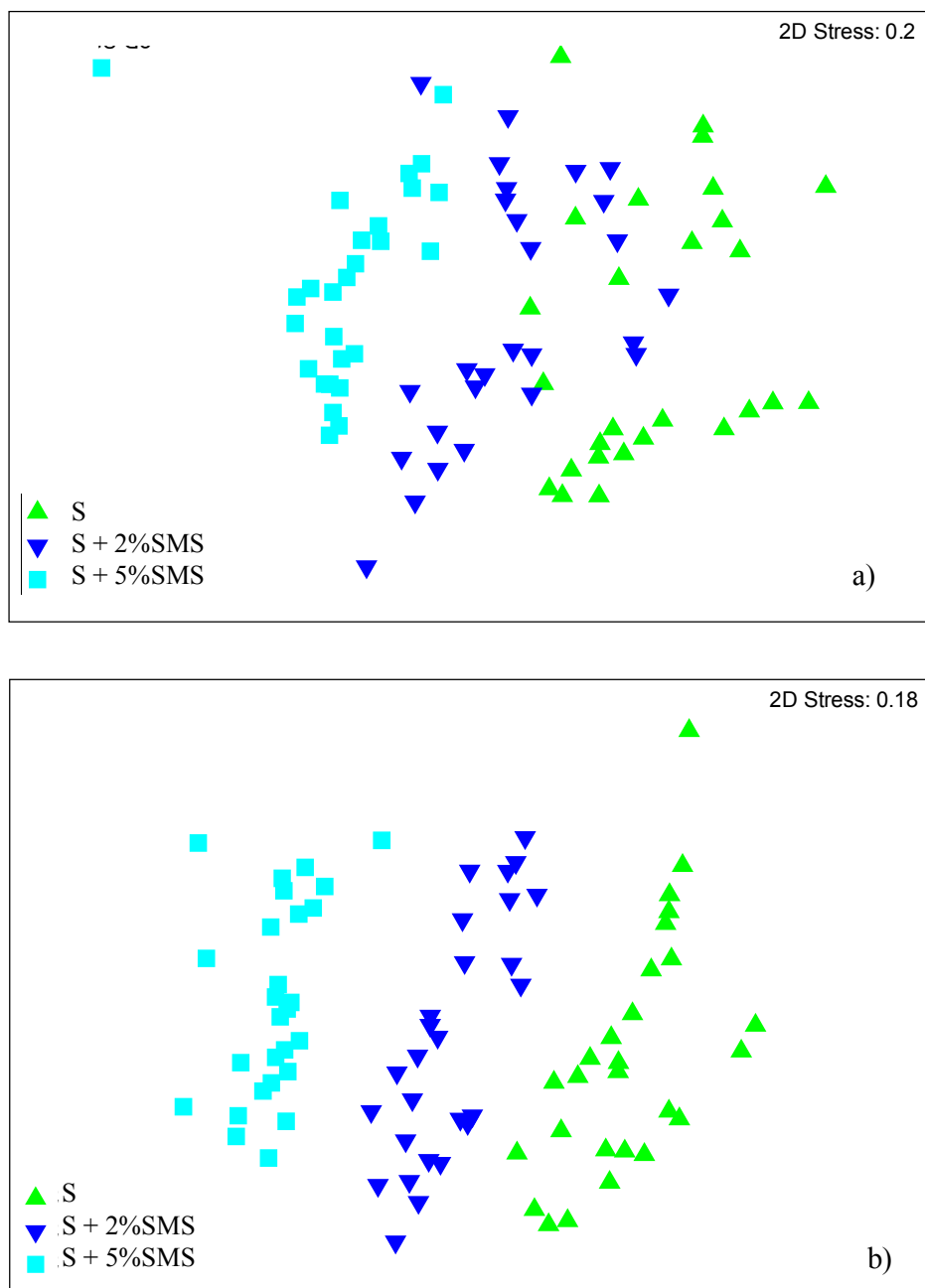


Fig. 4. Non-metric Multi-Dimensional Scaling of eukaryote community structure based on the azoxystrobin (a) and pirimicarb (b) samples from the three different soils.

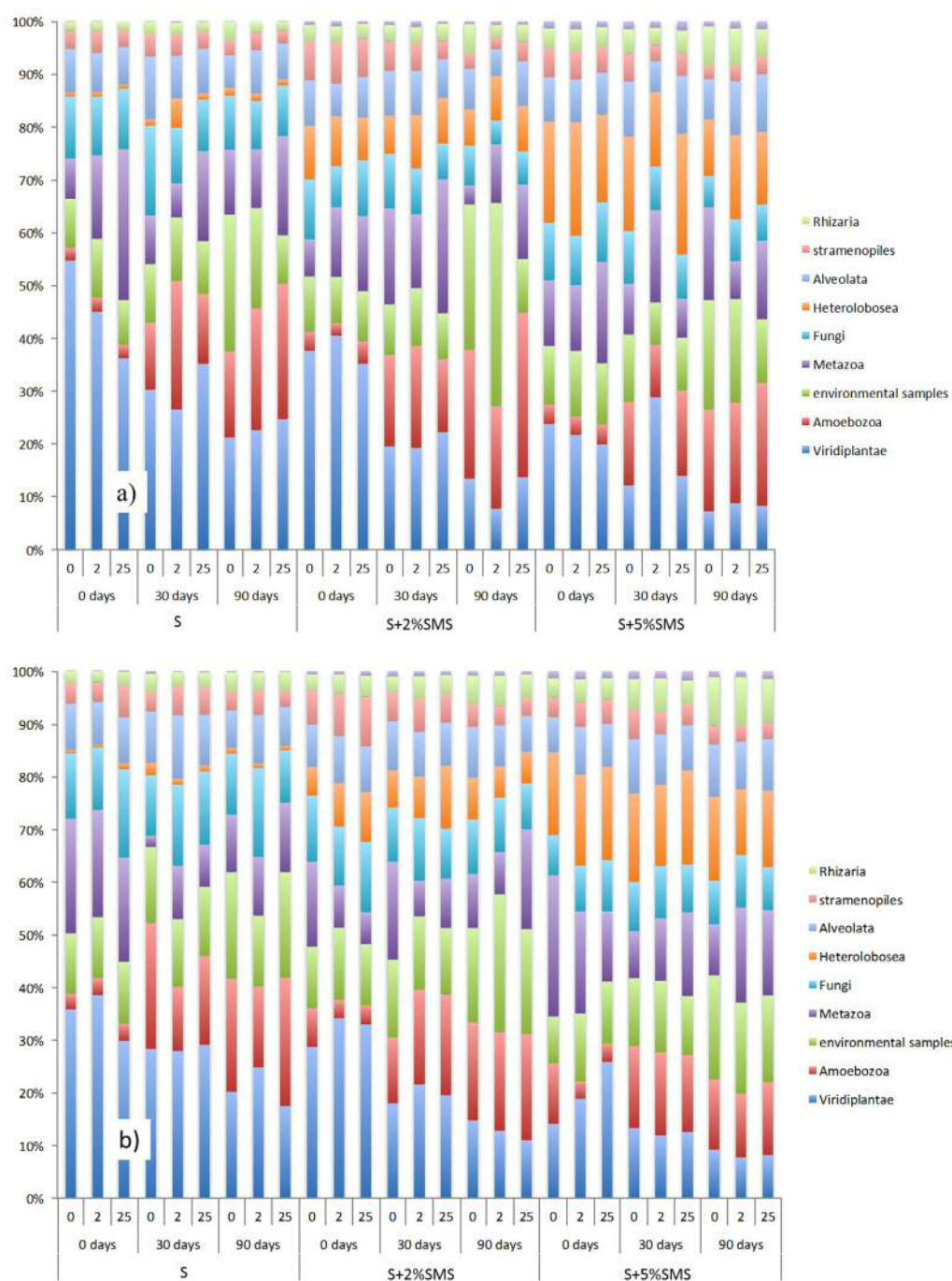


Fig. 5. The taxa richness and relative abundance found in every treatment (amendment doses, harvest time (0, 30 and 90 days) and pesticide concentration (0, 2 and 25 mg kg⁻¹)) for azoxystrobyn (a) and for pirimicarb (b).

Table 3. Analysis of similarity percentage (SIMPER) for azoxystrobin eukaryote communities. (Comparison between percentages of amendment)

| Soil comparisons A vs. B | Overall Average Dissimilarity | Five most influential taxa | Percent contribution to difference | Average percent abundance in class A±SEM | Average percent abundance in class B±SEM |
|--------------------------------|----------------------------------|---|--|--|--|
| S vs. S+2%SMS | 45.6 | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 16.2 | 12.7 ± 2.97 | 10.6 ± 2.46 |
| | | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 13.8 | 18.7 ± 2.29 | 9.49 ± 1.43 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 8.91 | 8.75 ± 1.35 | 10.1 ± 1.44 |
| | | environmental samples;uncultured eukaryote;Other;Other;Other | 8.71 | 11.1 ± 1.18 | 13.1 ± 2.15 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 4.98 | 0.53 ± 0.20 | 5.04 ± 0.38 |
| S vs. S+5%SMS | 56.3 | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 13.3 | 18.7 ± 2.29 | 4.23 ± 0.73 |
| | | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 12.3 | 12.7 ± 2.97 | 8.25 ± 1.99 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 8.25 | 0.53 ± 0.20 | 9.83 ± 0.58 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 6.48 | 8.75 ± 1.35 | 8.50 ± 1.12 |
| | | Viridiplantae;Chlorophyta;Chlorophyceae;Pseudomuriella;Pseudomuriella sp. Itas 9/21 14-1d | 5.77 | 6.66 ± 0.45 | 0.15 ± 0.02 |
| S+2%SMS vs. S+5%SMS | 43.9 | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 13.9 | 10.6 ± 2.46 | 8.25 ± 1.99 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 8.70 | 10.1 ± 1.44 | 8.50 ± 1.12 |
| | | environmental samples;uncultured eukaryote;Other;Other;Other | 8.65 | 13.1 ± 2.15 | 11.0 ± 0.92 |
| | | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 8.05 | 9.49 ± 1.43 | 4.23 ± 0.73 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 5.85 | 5.04 ± 0.39 | 9.83 ± 0.58 |

Table 4. Analysis of similarity percentage (SIMPER) for pirimicarb eukaryote communities. (Comparison between percentages of amendment)

| Soil comparisons | Overall Average Dissimilarity | Five most influential taxa | Percent contribution to difference | Average percent abundance in class A±SEM | Average percent abundance in class B±SEM |
|------------------------|----------------------------------|--|--|--|---|
| A vs. B | | | | | |
| S vs. S+2%SMS | 45.6 | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 14.4 | 11.4 ± 2.17 | 8.02 ± 1.73 |
| | | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 9.71 | 12.3 ± 1.09 | 9.28 ± 1.36 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 7.74 | 7.59 ± 1.06 | 8.25 ± 1.07 |
| | | environmental samples;uncultured eukaryote;Other;Other;Other | 6.25 | 12.4 ± 0.69 | 13.4 ± 1.03 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 4.92 | 0.24 ± 0.08 | 4.10 ± 0.55 |
| S vs. S+5%SMS | 56.3 | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 10.9 | 11.4 ± 2.17 | 10.2 ± 1.75 |
| | | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 8.87 | 12.3 ± 1.09 | 3.91 ± 0.74 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 7.96 | 0.24 ± 0.08 | 8.48 ± 0.44 |
| | | Viridiplantae;Chlorophyta;Chlorophyceae;Pseudomuriella;Pseudomuriella sp. Itas 9/21 14-1d | 6.08 | 6.39 ± 0.42 | 0.09 ± 0.01 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 5.89 | 7.59 ± 1.06 | 7.11 ± 1.76 |
| S+2%SMS vs. S+5%SMS | 43.9 | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 12.9 | 8.02 ± 1.73 | 10.2 ± 1.75 |
| | | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 8.94 | 9.28 ± 1.36 | 3.91 ± 0.74 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 7.71 | 8.25 ± 1.07 | 7.11 ± 1.76 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 6.62 | 4.10 ± 0.55 | 8.48 ± 0.44 |
| | | environmental samples;uncultured eukaryote;Other;Other;Other | 6.57 | 13.4 ± 1.03 | 11.4 ± 0.68 |

4 DISCUSSION

We found that the addition of SMS to soil increased soil microbial activity, and that this was associated with enhanced rates of pirimicarb degradation applied both high and low rates, and of azoxystrobin applied at the low dose. In the case of azoxystrobin applied at 25 mg kg⁻¹, higher immobilization of the pesticide in SMS amended soils resulted in reduced rates of degradation relative to the unamended soil.

Calculated DT₅₀ values of both pesticides are in accordance to those reported in previous works in unamended and amended soils (Bending et al., 2006, 2007; Kah et al., 2007; Howell et al. 2014; PPDB, 2015), and indicate that these pesticides are moderately persistent.

Previous researches have correlated the degradation of pesticides in soil with pH and OC. In particular azoxystrobin dissipation has been inversely correlated to the pH (Bending et al., 2006; Singh et al., 2010). SMS induced a decrease in soil pH and increased the OC content, which could have affected the interaction between SMS and the pesticide, thereby enhancing pesticide sorption and reducing bioavailability.

For both pesticides, bioavailability was higher when they were applied at a lower dose (2 mg kg⁻¹) than at a higher dose (25 mg kg⁻¹) as previously described (Sopeña and Bending 2013; Howell et al. 2014), and this could explain the lower DT₅₀ values found for both pesticides applied at a lower dose.

In our studies we found that DHA activity was stimulated by the SMS. These changes could reflect a source of labile carbon source provided by the amendments for the soil microbial community, or changes in microbial activity induced by modification to physicochemical properties of the soil, which may impact the pore structure of the soil and thereby aeration and water availability (Gao et al., 2015).

In addition, DHA was affected by the concentration of pirimicarb, decreasing after 90 days in the 25 mg kg⁻¹ treatment in SMS treated soils and this might reflect that the microbial community had a low resistance to pirimicarb application (Smith et al., 2000). Several studies (Pandey and Singh, 2006; Muñoz-Leoz et al., 2013) have also reported the increased sensitivity of soil DHA to other insecticides suggesting death or metabolic inhibition of a microbial fraction sensitive to these chemicals at high doses. However azoxystrobin did not have a significant effect on DHA activity at any time point. Moreover the dissipation studies show the persistence of azoxystrobin in the soil, indicating that there was not a direct link between azoxystrobin and DHA. Although decreased DHA has been shown previously in soils treated with this fungicide (Bending et al., 2007; Sopeña and Bending, 2013), this discrepancy could be due to adaptation towards more tolerant types of microbial communities, being not affected by pesticide exposure, in agreement with what we found in our sequencing results (see below).

Respiration increased in pesticide treated relative to untreated soils. Respiration has been extensively used as an ecophysiological index that can be used as a factor to evaluate environmental stress in microbial communities. Therefore, when the same biomass shifts to a higher respiratory activity it can reflect stress conditions, inducing more active metabolism and lower incorporation of organic carbon into the microbial biomass (Anderson and Domsch, 1985). In the current study respiration increased in a similar way following application of both pesticides, which did not correlate with the pesticide persistence, and was different for each compound. This could reflect either increased stress, or

microbial growth on biomass killed by pesticide application but probably not the use of the pesticide as carbon source (Marinozzi et al., 2013).

Sequence analysis of the eukaryote communities revealed that the concentration of amendment modified community composition. In fact the taxa richness was higher in the presence of SMS. These results are consistent with low resolution t-RFLP analysis carried out by Pérez-Piqueres et al. (2006).

Furthermore, within each soil treatment, there were significant changes in community composition over time. This supports evidence from DHA and respiration, which indicates that microbial activity showed variation across time points.

The predominant factor responsible for the change in the microbial structure was the input of amendment to soil (Vieublé-Gonod et al., 2009). Key taxa which responded to SMS application included microarthropods such collembola, which increased in the presence SMS as found for other organic amended soils (Jørgensen and Hedlund, 2013) and protists, which also increased, probably due to the higher microbial turnover and flux of macronutrients from microfauna in the treatments (Forge et al., 2003).

In contrast there was a decline in abundance of plant sequences. This reflects reduced relative abundance of plant sequences in the seed bank (Davies et al., 2013), which could reflect either a dilution by the amendment or a stimulated decomposition of plant debris following stimulated microbial activity after incorporation of the amendment.

In addition, one of the taxa more responsible for the changes when looking at fungal community was, as expected, *Agaricomycetes*, which increased with the amendment due to the presence of mushroom residues.

Interestingly, Howell et al. (2014) found a significant influence of azoxystrobin on microbial communities when profiling communities using RNA but not DNA extracts, suggesting a change in the active population rather than the total population. Additionally, it is important to notice that in those studies non-agricultural soils were used. Therefore in our agricultural soil, where pesticides have been used for decades, the endogenous microbial structures have probably been adapted to the stress caused by pesticide application, either by resistance or resilience capacities, as it has been previously suggested (Widenfalk et al., 2008; Howell et al., 2014).

In the current study eukaryote but not prokaryote communities were profiled. The data shows the power of micro-eukaryote sequencing to resolve taxa responding to agricultural management practices, thereby identifying indicators at a range of trophic levels, including fungi, protists and invertebrates. While the fungicide and insecticide used here could potentially have directly affect eukaryote communities, direct effects on bacterial communities could occur, associated with proliferation of degraders, and indirect effects on bacteria also could occur due to the competition with eukaryote communities (Adetutu et al., 2008; Arabet et al., 2014). However, clearly our results demonstrate that pesticides had little influence on the structure of micro-eukaryote communities, from any taxonomic group.

5 CONCLUSIONS

In this work we have studied the effect of the addition of azoxystrobin, pirimicarb and/or SMS to an agricultural soil at different time points on microbial communities by both broad scale and fine scale

analysis. Dissipation studies of both pesticides revealed their bioavailability in amended and unamended soils, which indicate their potential to alter microbial diversity. Additionally, we found that SMS facilitated the degradation of pirimicarb at both studied concentrations and of azoxystrobin at the lower concentration. Both DHA and respiration studies suggest a higher impact of SMS and time on communities than the much limited or absent effect of pesticides. In accordance both DHA and respiration decreased along incubation time. Similarly, Illumina MiSeq data revealed significant effects of both SMS amount and incubation time on the microbial communities, but no impact of pesticides. Therefore, the use of SMS as an organic amendment in agricultural soils could affect not only the dissipation kinetics of the pesticides but also the soil microbial communities.

Acknowledgements

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Supplementary Material

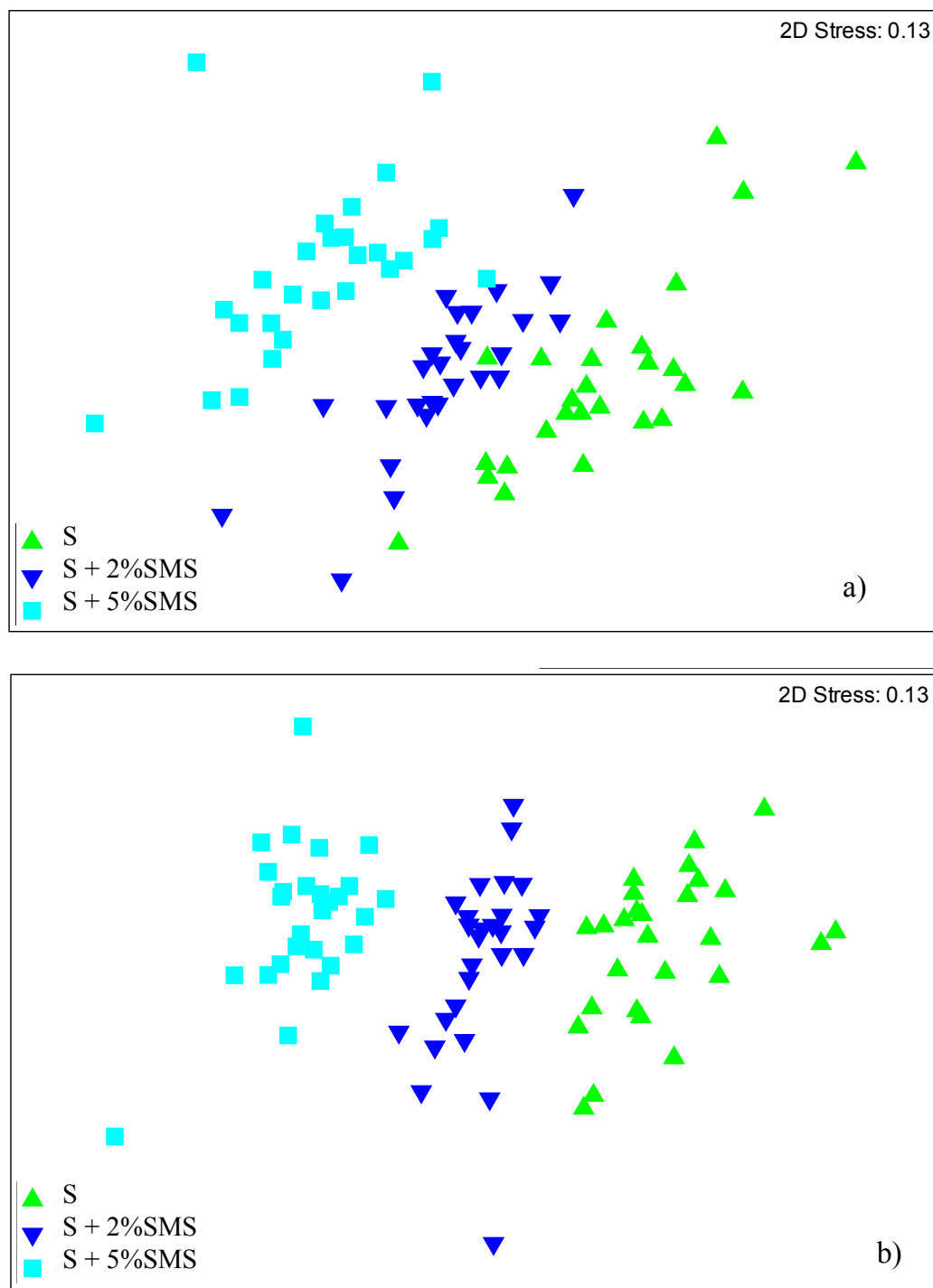


Fig. S1. Non-metric Multi-Dimensional of fungal community structure based on the azoxystrobin (a) and pirimicarb (b) fungal samples from the three different soils.

Table S1. Analyses of similarity (ANOSIM) evaluating the variation of eukaryote communities (Comparison of harvest time in each type of soil)

| S | Azoxystrobin | | Pirimicarb | |
|------------------|--------------|-------|------------|-------|
| | R | p | R | p |
| 0 days, 30 days | 0.124 | 0.002 | 0.631 | 0.001 |
| 0 days, 90 days | 0.683 | 0.001 | 0.695 | 0.001 |
| 30 days, 90 days | 0.206 | 0.026 | 0.362 | 0.006 |
| S + 2%SMS | R | p | R | p |
| 0 days, 30 days | 0.613 | 0.001 | 0.615 | 0.001 |
| 0 days, 90 days | 0.905 | 0.001 | 0.929 | 0.001 |
| 30 days, 90 days | 0.366 | 0.006 | 0.381 | 0.001 |
| S + 5%SMS | R | p | R | p |
| 0 days, 30 days | 0.572 | 0.001 | 0.483 | 0.003 |
| 0 days, 90 days | 0.848 | 0.001 | 0.750 | 0.001 |
| 30 days, 90 days | 0.424 | 0.001 | 0.458 | 0.001 |

Table S2. Analyses of similarity (ANOSIM) evaluating the variation of fungal communities. (Comparison of percentage of amendment)

| Soils comparisons | Azoxystrobin | | Pirimicarb | |
|-------------------|--------------|-------|------------|-------|
| | R | p | R | p |
| S, S+2%SMS | 0.306 | 0.001 | 0.510 | 0.001 |
| S, S+5%SMS | 0.770 | 0.001 | 0.966 | 0.001 |
| S+2%SMS, S+5%SMS | 0.432 | 0.001 | 0.649 | 0.001 |

Table S3. Analyses of similarity (ANOSIM) evaluating the variation of fungal communities. (Comparison of harvest time in each type of soil)

| S | Azoxystrobin | | Pirimicarb | |
|------------------|--------------|-------|------------|-------|
| | R | p | R | p |
| 0 days, 30 days | 0.135 | 0.034 | 0.477 | 0.002 |
| 0 days, 90 days | 0.322 | 0.001 | 0.439 | 0.001 |
| 30 days, 90 days | 0.163 | 0.025 | 0.219 | 0.004 |
| S + 2%SMS | R | p | R | p |
| 0 days, 30 days | 0.352 | 0.001 | 0.239 | 0.001 |
| 0 days, 90 days | 0.536 | 0.001 | 0.430 | 0.001 |
| 30 days, 90 days | 0.180 | 0.004 | 0.217 | 0.007 |
| S + 5%SMS | R | p | R | p |
| 0 days, 30 days | 0.039 | 0.169 | 0.319 | 0.001 |
| 0 days, 90 days | 0.728 | 0.001 | 0.413 | 0.001 |
| 30 days, 90 days | 0.496 | 0.001 | 0.368 | 0.001 |

Table S4. Analysis of similarity percentage (SIMPER) for azoxystrobin eukaryote communities. (Comparison between harvest time)

| Soil comparisons | Overall | | Percent | Average percent | Average percent |
|---------------------------|---------------|---|-----------------|-------------------|-------------------|
| A vs. B | Average | Five most influential taxa | contribution to | abundance in | abundance in |
| | Dissimilarity | | difference | class A \pm SEM | class B \pm SEM |
| 0 days vs. 30 days | 46.1 | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 14.8 | 11.3 \pm 2.28 | 11.0 \pm 2.71 |
| | | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 14.4 | 18.9 \pm 2.36 | 7.82 \pm 1.23 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 11.1 | 1.28 \pm 0.18 | 11.5 \pm 0.95 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 5.77 | 5.85 \pm 1.01 | 5.32 \pm 0.80 |
| | | Viridiplantae;Chlorophyta;Chlorophyceae;Pseudomuriella;Pseudomuriella sp. Itas 9/21 14-1d | 4.62 | 3.74 \pm 0.61 | 4.52 \pm 0.93 |
| 0 days vs. 90 days | 52.7 | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 13.7 | 18.9 \pm 2.36 | 5.72 \pm 1.02 |
| | | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 12.7 | 11.3 \pm 2.28 | 9.20 \pm 2.55 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 12.5 | 1.28 \pm 0.18 | 14.5 \pm 0.76 |
| | | environmental samples;uncultured eukaryote;Other;Other;Other | 9.91 | 8.57 \pm 0.34 | 18.2 \pm 2.07 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 4.87 | 5.85 \pm 1.01 | 4.23 \pm 0.67 |
| 30 days vs. 90 days | 43.5 | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 15.7 | 11.0 \pm 2.71 | 9.20 \pm 2.55 |
| | | environmental samples;uncultured eukaryote;Other;Other;Other | 12.1 | 8.44 \pm 0.37 | 18.2 \pm 2.07 |
| | | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 7.20 | 7.82 \pm 1.23 | 5.72 \pm 1.02 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 6.30 | 11.5 \pm 0.95 | 14.5 \pm 0.76 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 5.06 | 5.32 \pm 0.08 | 4.23 \pm 0.67 |

Table S5. Analysis of similarity percentage (SIMPER) for pirimicarb eukaryote communities. (Comparison between harvest time)

| Soil comparisons | Overall | | Percent | Average percent | Average percent |
|---------------------------|---------------|---|-----------------|-------------------|-------------------|
| A vs. B | Average | Five most influential taxa | contribution to | abundance in | abundance in |
| | Dissimilarity | | difference | class A \pm SEM | class B \pm SEM |
| 0 days vs. 30 days | 41.7 | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 14.8 | 13.5 \pm 2.03 | 6.81 \pm 1.88 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 11.6 | 1.97 \pm 0.72 | 10.9 \pm 0.72 |
| | | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 10.4 | 13.5 \pm 1.46 | 7.24 \pm 0.91 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 5.37 | 4.24 \pm 0.73 | 4.61 \pm 0.85 |
| | | Viridiplantae;Chlorophyta;Chlorophyceae;Pseudomuriella;Pseudomuriella sp. Itas 9/21 14-1d | 4.26 | 3.49 \pm 0.60 | 3.70 \pm 0.64 |
| 0 days vs. 90 days | 44.7 | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 12.2 | 13.5 \pm 2.03 | 9.34 \pm 1.57 |
| | | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 10.9 | 13.5 \pm 1.46 | 4.76 \pm 0.68 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 9.85 | 1.97 \pm 0.72 | 10.0 \pm 0.52 |
| | | environmental samples;uncultured eukaryote;Other;Other;Other | 7.61 | 9.83 \pm 0.40 | 16.4 \pm 0.80 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 4.65 | 4.24 \pm 0.73 | 3.98 \pm 0.73 |
| 30 days vs. 90 days | 36.2 | Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona | 13.5 | 6.81 \pm 1.88 | 9.34 \pm 1.57 |
| | | environmental samples;uncultured eukaryote;Other;Other;Other | 8.48 | 11.0 \pm 0.57 | 16.4 \pm 0.80 |
| | | Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses | 6.62 | 7.24 \pm 0.91 | 4.76 \pm 0.68 |
| | | Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoeba sp. ATCC 50324 | 6.01 | 4.61 \pm 0.85 | 3.98 \pm 0.73 |
| | | Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae | 5.03 | 10.9 \pm 0.72 | 10.0 \pm 0.52 |

Table S6. Analysis of similarity percentage (SIMPER) for azoxystrobin fungal communities. (Comparison between percentages of amendment)

| Soil comparisons A vs. B | Overall Average Dissimilarity | Five most influential taxa | Percent contribution to difference | Average percent abundance in class A \pm SEM | Average percent abundance in class B \pm SEM |
|-----------------------------|-------------------------------------|--|--|--|---|
| S vs. S+2%SMS | 40.4 | Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes | 15.2 | 1.58 \pm 0.62 | 0.45 \pm 0.14 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 9.52 | 1.91 \pm 0.14 | 1.38 \pm 0.08 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes | 8.41 | 1.01 \pm 0.12 | 0.56 \pm 0.07 |
| | | Fungi;Fungi incertae sedis;Basal fungal lineages;unclassified zygomycetes;zygomycete sp. AM-2008a | 5.91 | 0.82 \pm 0.13 | 0.39 \pm 0.03 |
| | | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 4.88 | 0.14 \pm 0.01 | 0.49 \pm 0.06 |
| S vs. S+5%SMS | 53.7 | Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes | 11.8 | 1.58 \pm 0.62 | 0.57 \pm 0.17 |
| | | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 10.4 | 0.14 \pm 0.01 | 1.25 \pm 0.26 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 9.90 | 1.91 \pm 0.14 | 0.89 \pm 0.06 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes | 7.64 | 1.01 \pm 0.12 | 0.21 \pm 0.2 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes | 7.51 | 0.10 \pm 0.01 | 0.84 \pm 0.05 |
| S+2%SMS vs. S+5%SMS | 41.1 | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 12.1 | 0.49 \pm 0.06 | 1.25 \pm 0.26 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 8.86 | 1.38 \pm 0.08 | 0.89 \pm 0.06 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes | 6.75 | 0.43 \pm 0.3 | 0.84 \pm 0.05 |
| | | Fungi;Fungi incertae sedis;Basal fungal lineages;Mucoromycotina;Mucorales | 6.74 | 0.37 \pm 0.04 | 0.77 \pm 0.05 |
| | | Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes | 6.41 | 0.45 \pm 0.14 | 0.57 \pm 0.17 |

Table S7. Analysis of similarity percentage (SIMPER) for pirimicarb fungal communities. (Comparison between percentages of amendment)

| Soil comparisons A vs. B | Overall Average Dissimilarity | Five most influential taxa | Percent contribution to difference | Average percent abundance in class A \pm SEM | Average percent abundance in class B \pm SEM |
|-----------------------------|-------------------------------------|--|--|--|---|
| S vs. S+2%SMS | 37.2 | Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes | 12.1 | 1.64 \pm 0.21 | 0.62 \pm 0.05 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 11.9 | 2.45 \pm 0.26 | 1.54 \pm 0.07 |
| | | Fungi;Chytridiomycota;environmental samples;uncultured Chytridiomycota;Other | 6.96 | 1.12 \pm 0.09 | 0.55 \pm 0.04 |
| | | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 5.65 | 0.13 \pm 0.01 | 0.63 \pm 0.07 |
| | | Fungi;Fungi incertae sedis;Basal fungal lineages;unclassified zygomycetes;zygomycete sp. AM-2008a | 5.55 | 1.01 \pm 0.07 | 0.57 \pm 0.03 |
| S vs. S+5%SMS | 54.4 | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 12.5 | 2.45 \pm 0.26 | 0.94 \pm 0.07 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes | 11.8 | 1.64 \pm 0.21 | 0.19 \pm 0.01 |
| | | Fungi;Chytridiomycota;environmental samples;uncultured Chytridiomycota;Other | 9.44 | 0.13 \pm 0.01 | 1.24 \pm 0.14 |
| | | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 7.1 | 1.12 \pm 0.09 | 0.27 \pm 0.02 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes | 6.97 | 0.07 \pm 0.08 | 0.89 \pm 0.05 |
| S+2%SMS vs. S+5%SMS | 37.5 | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 10.5 | 0.63 \pm 0.07 | 0.63 \pm 0.07 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 9.44 | 1.54 \pm 0.07 | 1.54 \pm 0.07 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes | 5.81 | 0.62 \pm 0.05 | 0.62 \pm 0.05 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes | 7.72 | 0.51 \pm 0.03 | 0.51 \pm 0.03 |
| | | Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes | 5.47 | 0.73 \pm 0.16 | 0.58 \pm 0.03 |

Table S8. Analysis of similarity percentage (SIMPER) for azoxystrobin fungal communities. (Comparison between harvest time)

| Soil comparisons A vs. B | Overall Average Dissimilarity | Five most influential taxa | Percent contribution to difference | Average percent abundance in class A \pm SEM | Average percent abundance in class B \pm SEM |
|-----------------------------|-------------------------------------|--|--|--|---|
| 0 days vs. 30 days | 39.9 | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 10.97 | 0.81 \pm 0.15 | 0.75 \pm 0.26 |
| | | Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes | 10.05 | 0.64 \pm 0.19 | 1.00 \pm 0.51 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 9.04 | 1.53 \pm 0.10 | 1.52 \pm 0.16 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes | 8.54 | 0.97 \pm 0.14 | 0.48 \pm 0.06 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes | 5.47 | 0.43 \pm 0.06 | 0.54 \pm 0.07 |
| 0 days vs. 90 days | 44.6 | Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes | 10.9 | 0.64 \pm 0.19 | 0.96 \pm 0.04 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes | 8.93 | 0.97 \pm 0.14 | 0.30 \pm 0.04 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 8.76 | 1.53 \pm 0.10 | 1.13 \pm 0.09 |
| | | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 8.38 | 0.81 \pm 0.15 | 0.31 \pm 0.04 |
| | | Fungi;Chytridiomycota;environmental samples;uncultured Chytridiomycota;Other | 4.98 | 0.59 \pm 0.05 | 0.23 \pm 0.02 |
| 30 days vs. 90 days | 43.8 | Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes | 13.2 | 1.00 \pm 0.51 | 0.96 \pm 0.04 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 9.6 | 1.52 \pm 0.16 | 1.13 \pm 0.09 |
| | | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 8.03 | 0.75 \pm 0.26 | 0.31 \pm 0.04 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes | 5.99 | 0.54 \pm 0.07 | 0.48 \pm 0.06 |
| | | Fungi;Fungi incertae sedis;Basal fungal lineages;Mucoromycotina;Mucorales | 5.12 | 0.59 \pm 0.06 | 0.37 \pm 0.04 |

Table S9. Analysis of similarity percentage (SIMPER) for pirimicarb fungal communities. (Comparison between harvest time)

| Soil comparisons | Overall | | Percent | Average percent | Average percent |
|---------------------------|---------------|--|-----------------|-------------------|-------------------|
| A vs. B | Average | Five most influential taxa | contribution to | abundance in | abundance in |
| | Dissimilarity | | difference | class A \pm SEM | class B \pm SEM |
| 0 days vs. 30 days | 37.1 | Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes | 12.17 | 1.31 \pm 0.25 | 0.58 \pm 0.09 |
| | | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 10.8 | 0.94 \pm 0.17 | 0.64 \pm 0.09 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 9.97 | 1.40 \pm 0.11 | 1.79 \pm 0.15 |
| | | Fungi;Chytridiomycota;environmental samples;uncultured Chytridiomycota;Other | 6.88 | 0.70 \pm 0.09 | 0.77 \pm 0.11 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes | 5.40 | 0.44 \pm 0.06 | 0.57 \pm 0.08 |
| 0 days vs. 90 days | 41.9 | Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes | 10.9 | 1.31 \pm 0.25 | 0.56 \pm 0.08 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 10.0 | 1.40 \pm 0.11 | 1.73 \pm 0.09 |
| | | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 9.28 | 0.94 \pm 0.17 | 0.41 \pm 0.06 |
| | | Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes | 5.53 | 0.73 \pm 0.11 | 0.62 \pm 0.13 |
| | | Fungi;Chytridiomycota;environmental samples;uncultured Chytridiomycota;Other | 5.02 | 0.70 \pm 0.09 | 0.46 \pm 0.05 |
| 30 days vs. 90 days | 37.6 | Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes | 13.1 | 1.79 \pm 0.15 | 1.73 \pm 0.09 |
| | | Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes | 6.31 | 0.64 \pm 0.09 | 0.41 \pm 0.06 |
| | | Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes | 6.07 | 0.57 \pm 0.08 | 0.47 \pm 0.07 |
| | | Fungi;Chytridiomycota;environmental samples;uncultured Chytridiomycota;Other | 6.04 | 0.77 \pm 0.11 | 0.46 \pm 0.05 |
| | | Fungi;Fungi incertae sedis;Basal fungal lineages;unclassified zygomycetes;zygomycete sp. AM-2008a | 5.86 | 0.67 \pm 0.06 | 0.70 \pm 0.10 |

Imagen de portada:
Vincent van Gogh
El viñedo rojo cerca de Arlés
Museo Pushkin, Moscú, Rusia



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